STUDIES ON PHOSPHOLIPASES PRODUCED BY *CORTICIUM CENTRIFUGUM*

SATOSHI UEHARA

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CONTENTS

ABBREVIATIONS

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PI, phosphatidylinositol

PG, phosphatidylglycerol

CL, cardiolipin

PS, phosphatidylserine

LPC, lysophosphatidylcholine

GPC, glycerylphosphorylcholine

FFA, free fatty acid

PLase, phospholipase

PLase A, phospholipase A

PLase B, phospholipase B

PLase C, phospholipase C

PLase D, phospholipase D

LPLase, lysophospholipase

LPC-LPC acyltransferase, lysolecithin-lysolecithin acyltransferase

TLC, thin-layer chromatography

EDTA, ethylenediamine tetraacetic acid

PPO, 2,5-diphenyloxazole

POPOP, 1,4-bis [2-(5-phenyloxazolyl)]-benzene

CHAPTER I

INTRODUCTION

The mold, *Corticium centrifugum* is an aggressive plant pathogen, belonging to Basidiomycetes, and has been studied for a long time in the field of plant pathology.[1] Enzyme preparations from *C. centrifugum*, on the other hand, have been used in the food industry as a fruit juice clarifying agent,[2-3] especially for the grape, by utilizing its pectinase activity and as a cell wall lyser[4-5] because of its glucanase and protease activities.

In regard to lipolytic enzymes produced by this mold, there were previously only three reports on PLase B from *Sclerotium rolfsii*, another name of *C. centrifugum*, by Tseng *et al.*,[6-8] and it was considered that PLase B was the only PLase of this mold. Recently Hasegawa *et al.*[9] reported the presence of PLase A and LPLase in addition to PLase B in *C. centrifugum* IAM 9028. The presence of these PLases is interesting from the viewpoint of the mechanism of invasion of this mold upon plant tissues[6-8] and of utilization of enzymes.

The problem which has been argumented in host-pathogen physiology relates to the mechanism of the killing of plant cells which accompanies tissue maceration.[10-18] PLases which directly attack phospholipids in cell membranes have been noticed as a killing substance of a cell,[7] and it was shown by Tseng *et al.*[6] that the ability to produce PLases was widely distributed over various plant

pathogens. But the kinds of PLases identified were only two, PLase B from *Sclerotium rolfsii*[7] and *Botrytis cinerea*[19] and PLase C from *Erwinia carotovora*,[20] and there were no reports of highly purified PLases besides.

At the present time little knowledge has been gained of PLases from a mold.

From above reasons, in order to clarify the PLases produced by *C. centrifugum*, each PLase was separately purified and investigated of its properties by using *Corticium centrifugum* IAM 9028, of which culture broth had various PLases with strong activities.[9]

In the present thesis, the purification and properties of PLases A_1 and B and LPLase 1,2 produced by *C. centrifugum* IAM 9028 are described. The phenomenon that LPC-LPC acyltransferase activity comes into the appearance in LPLase is also described. And finally the characteristic properties and relation of PLases produced by *C. centrifugum* IAM 9028 are described.

CHAPTER II

PURIFICATION AND PROPERTIES OF PHOSPHOLIPASE A_1 PRODUCED BY *CORTICIUM CENTRIFUGUM*

Hasegawa *et al.*[9] reported the presence of PLases A and B and LPLase in *Corticium centrifugum* IAM 9028. This was the first report that showed the presence of PLase A in plant pathogens and a mold. The detailed properties and the reaction type of this enzyme, however, have not been shown as yet. In the present study, a strong PLase A activity was found in the culture broth of this mold as a result of the examination using Triton X-100 in the assay condition.

This Chapter describes the purification and properties of PLase A_1 produced by *C. centrifugum* IAM 9028.

In this Chapter, the terms PLase A_1[21] and LPLase 1[22] are used for the enzymes that catalyze the hydrolysis of the fatty acids at position 1 of the glycerol moiety of glycerophospholipids and lysophospholipids, respectively.

Materials and Methods

Enzyme. The mold, *Corticium centrifugum* IAM 9028, was cultured at 28°C for 72 hr in a medium, consisting of 4% of heat-treated brewer's yeast, 0.1% of KH_2PO_4, 0.05% of KCl, 0.05% of $MgSO_4 \cdot 7H_2O$ and 0.001% of $FeSO_4 \cdot 7H_2O$. The culture broth, about 60 liters, was neutralized by 2N NaOH, and the mycelia were eliminated

by continuous centrifugation. The supernatant was filtered through a pulp layer. The filtrate was added ammonium sulfate until 100% saturation was attained, and the mixture was stirred overnight. The precipitate was collected by centrifugation and dialyzed against 2 mM phosphate buffer, pH 7.1, for 4 hr. The dialyzed solution, about 2 liters, was desalted by gel filtration with a Sephadex G-25 column, which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. The elution was carried out with the same buffer, and the desalted solution was stored at -20°C. The purification of PLase A was accomplished with this desalted solution.

Substrate. PC (Type III-E, egg yolk) and triolein were purchased from Sigma Chem. Co., and PE (egg), PG (egg lecithin), CL (beef heart) and PS (bovine brain) were purchased from Serdary Res. Lab. Inc., and were purified by silicic acid column chromatography. 1-Acyl-LPC was prepared by the action of *Crotalus adamanteus* PLase A_2 (Sigma Chem. Co.) on PC by the procedure of Hanahan,[23] and purified by silicic acid column chromatography. 1-Acyl-LPC was generally used as a substrate for the assay of LPLase activity. 2-Acyl-LPC was prepared by the method as described by Nishijima *et al.*[24] using *Rhizopus delemer* lipase (Seikagaku-kogyo, pure grade).

Enzyme assay. The standard incubation mixture for determination of PLase A activity contained 0.5 ml of ultrasonic dispersion of PC (2 μmoles), which was sonicated with an ultrasonic oscillator (Branson Sonifier), 10 kHz, for 2 min in 0.1 M acetate

buffer, pH 4.0, containing 0.4% Triton X-100, 0.4 ml of 0.1 M acetate buffer, pH 4.0, and 0.1 ml of enzyme solution in a total volume of 1 ml. Incubation was usually carried out at 30°C for 6 min with shaking at a rate of 120 times per min. The reaction was stopped by the addition of 2.5 ml of methanol and 1.25 ml of chloroform, immediately followed by chilling at 0°C. Into the tube were added 1.25 ml of chloroform and 1.25 ml of water in accordance with the method of Bligh and Dyer.[25] The tube was shaken for an aliquot time at each additional step. The tube was centrifuged for 5 min, and a 2 ml aliquot of the chloroform phase was taken for the determination of decrease in fatty acyl ester group.[26]

The standard incubation mixture for determination of LPLase activity contained 0.5 ml of 0.1 M acetate buffer solution of LPC (2 μmoles), 1.4 ml of 0.1 M acetate buffer, pH 4.0, and 0.1 ml of enzyme solution in a total volume of 2 ml. Incubation was carried out at 30°C for 10 min with shaking at a rate of 120 times per min. The reaction was stopped by the addition of 5 ml of methanol and by heating at 80°C for 1 min. After cooling, to the tube were added 2.5 ml of chloroform, twice, and 2.5 ml of water in accordance with the method of Bligh and Dyer.[25] The tube was shaken for an aliquot time at each additional step. The tube was centrifuged for 5 min, and the upper layer was removed by suction. One ml of the chloroform phase was taken as a sample for TLC, and the residual chloroform phase (4 ml) was used for the determination of FFA by

the method of Duncombe[27] with palmitic acid as standard, as described by Satouchi *et al.*[28] Lipase activity was assayed as follows: the incubation mixture contained 0.5 ml of ultrasonic dispersion of triolein (2 μmoles), which was sonicated with an ultrasonic oscillator in 0.1 M acetate buffer, pH 4.0, 1.4 ml of 0.1 M acetate buffer, pH 4.0, and 0.1 ml of enzyme solution in a total volume of 2 ml. Incubation was carried out at 30°C for 30 min with shaking at a rate of 120 times per min. The rest of the assay procedure was the same as that of LPLase activity.

One unit each of PLase A and LPLase activities was defined as the amount of the corresponding enzyme which liberated 1 μeq of ester bond or 1 μmole of FFA per min under the standard assay conditions.

Positional specificity of PLase A. In order to investigate the positional specificity of PLase A, the mass spectra[29] of trimethylsilyl ether derivatives of 1- and 2-monoglycerides were analyzed by mass chromatography. After the enzyme reaction with purified or crude PLase A, a lyophilized preparation of the desalted solution of the precipitate from the culture filtrate by saturation with ammonium sulfate, the reaction products were partitioned between chloroform and aqueous methanol phases by the procedure of Bligh and Dyer.[25] An aliquot of chloroform phase was taken and evaporated. To the residue, 0.5 ml of 0.1 M borate buffer, pH 6.5, and 0.5 ml of the same buffer containing 1 mg of

Bacillus cereus PLase C (Calbiochem) were added. The mixture was incubated at 30°C for 30 min. After the incubation, the lipid fraction was extracted by the method of Bligh and Dyer.[25] The chloroform phase was concentrated and spotted on a boric acid-impregnated Silica Gel G plate.[30] TLC was carried out with chloroform-acetone (96:4, v/v), and the part of monoglycerides was scraped. The monoglycerides were extracted three times by the method of Bligh and Dyer.[25] An aliquot of the chloroform phase was taken and evaporated off. This residue was trimethylsilylated and subjected to mass chromatography. The molecular species of monoglycerides were analyzed by mass chromatography, and the quantification of each of the 1- and 2-monoglycerides was carried out by gas chromatography. Mass chromatography was carried out on a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer equipped with a MS-PAC 300 DGB data system. A 3 mm x 2 m glass column packed with 3% SE-52 on Chromosorb W, 60-80 mesh, acid washed and silanized, was used. The column oven temperature was programed from 200 to 270°C at a rate of 3°C/min. The ion source, molecular separator and injection port were kept at 290°C, 280°C and 280°C, respectively. The ionizing energy, accelerating voltage and trap current were 22 eV, 3.5 kV and 60 μA, respectively. Mass spectra were measured at 10-second intervals from m/e 30 to m/e 600 at a scan speed of 8.

Thin-layer chromatography. The reaction products which were extracted in the chloroform phase by the procedure of Bligh and Dyer[25] were analyzed by TLC, using Silica Gel G plates (Merck) with chloroform-methanol-water (80:35:5, v/v/v) as solvent.[9] Spots were visualized by iodine vapor.

Isoelectric focusing. Isoelectric focusing was carried out in a 30 ml apparatus devised by Doi *et al.*,[31] in accordance with the method of Vesterberg.[32] The electrophoresis was performed with a carrier of pH 2.5-6.0 at 500-800 V for 46-50 hr at 0-4°C. After the electrophoretic run, the ampholine solution was fractionated to 1 ml, followed by measurement of pH and the activity.

Disc electrophoresis. Disc electrophoresis was carried out in 7.5% gel for 90 min with a current of 2 mA/tube at 4°C in accordance with the method of Williams *et al.* (pH 8.0).[33] Sodium dodecyl sulfate disc electrophoresis was carried out in 10% gel for 5 hr with a current of 8 mA/tube at 4°C in accordance with the method of Weber *et al.*[34] Protein bands were stained with Coomassie Brilliant Blue R-250.

Estimation of molecular weight. The molecular weight of the enzyme was determined by gel filtration as described by Andrews.[35]

Determination of protein concentration. Protein concentration was determined by the method of Lowry *et al.*,[36] with bovine serum albumin as the standard.

Results

Purification of PLase A

Step 1. First DEAE-Sephadex column chromatography. The desalted solution was applied to a DEAE-Sephadex A-25 column (Fig. 1). PLase A activity was eluted with 0.45 M NaCl. The combined active fractions (tube Nos. 62-81) were concentrated and dialyzed against 0.05 M Tris-HCl buffer, pH 7.2, with a collodion-bag. LPLase activity was eluted with 0.25 and 0.45 M NaCl.

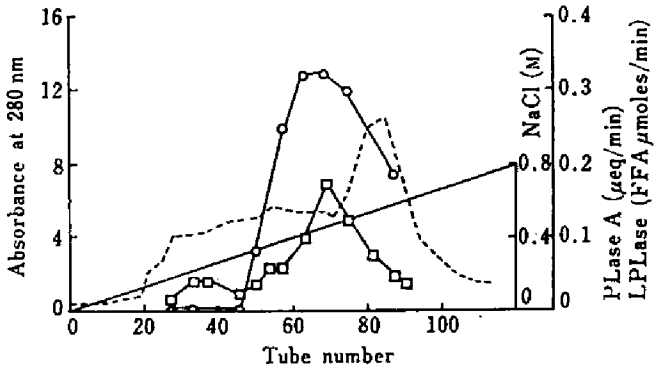

Fig. 1. First DEAE-Sephadex Column Chromatography of PLase A from *C. centrifugum*.

The desalted solution was applied to a DEAE-Sephadex A-25 column (2.6 x 27.5 cm), which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. After washing inactive proteins with the same buffer, elution was carried out at a rate of 50 ml/hr with a linear gradient of NaCl concentration from zero to 0.8 M in the same buffer, and 10 ml fractions were collected.

- - - - -, absorbance at 280 nm; O—O, PLase A activity; □—□, LPLase activity; ———, NaCl concentration.

Step 2. Second DEAE-Sephadex column chromatography. The dialyzed solution was applied to a DEAE-Sephadex A-25 column (2.6 x 21.5 cm), which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. After eluting inactive proteins with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl, and then with the same buffer containing 0.2 M NaCl, the elution, at a rate of 40 ml/hr, was carried out with a linear gradient of NaCl concentration from 0.2 M to 0.65 M in the same buffer, and 10 ml fractions were collected. The combined active fractions were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.1, with a collodion-bag.

Step 3. Hydroxylapatite column chromatography. The dialyzed solution was applied to a hydroxylapatite column (2.6 x 14.5 cm), which was composed of hydroxylapatite and cellulose (1:1, v/v), and was equilibrated with 10 mM phosphate buffer, pH 7.1. Elution was carried out at a rate of 60 ml/hr with a stepwise increase in buffer concentration (10, 50 and 100 mM), and 5 ml fractions were collected. The activity was eluted with 50 mM phosphate buffer. The combined active fractions were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.1, containing 0.1 M NaCl, with a collodion-bag.

Step 4. First Sephadex G-200 column chromatography. The dialyzed solution was applied to a Sephadex G-200 column (2.6 x 92 cm), which was equilibrated with 10 mM phosphate buffer, pH 7.1,

containing 0.1 M NaCl. Elution was carried out at a rate of 24 ml/hr with the same buffer, and 6 ml fractions were collected. The combined active fractions were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.1.

Step 5. Isoelectric focusing. The dialyzed solution was applied to an isoelectric focusing column. The electrophoresis was performed as described in Materials and Methods. The activity was found at about pH 3.3. The combined active fractions were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.0, containing 0.4 M KCl, with a collodion-bag.

Step 6. Second Sephadex G-200 column chromatography. The dialyzed solution was applied to a Sephadex G-200 column (1.6 x 92 cm), which was equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.4 M KCl. Elution was carried out at a rate of 10 ml/hr with the same buffer, and 2 ml fractions were collected. The combined active fractions were concentrated, dialyzed against 10 mM phosphate buffer, pH 7.1, and stored at -20°C.

The results of purification by the above procedures are summarized in Table I. The final preparation showed a 78.6-fold increase in specific activity of PLase A. The ratio of PLase A activity was constant at about 7.2 after the first DEAE-Sephadex column chromatography. The hydrolysis reactions of both activities showed almost linear rates up to at least 10 min incubation under

TABLE I. SUMMARY OF PURIFICATION OF PLASE A AND LPLASE

Procedure	Protein (mg)	Total activity (unit)		Specific activity (units/mg)		PLase A/ LPLase
		PLase A	LPLase	PLase A	LPLase	
$(NH_4)_2SO_4$ ppt. (100% satn.)	10000	156000	25118	11.2	1.8	6.2
1st DEAE-Sephadex	1288	84000	11368	65.2	8.8	7.4
2nd DEAE-Sephadex	144.9	33952	4697	234.3	32.4	7.2
Hydroxylapatite	25.9	20584	2855	794.8	110.2	7.2
1st Sephadex G-200	16.6	13733	1892	827.3	114.0	7.3
Isoelectric focusing	4.1	3463	482	840.5	117.0	7.2
2nd Sephadex G-200	3.1	2712	375	880.6	121.7	7.2

the standard assay conditions. The homogeneity of purified enzyme was about 90% on disc electrophoresis (pH 8.0), and both PLase A and LPLase activities were found only at the same position on a main protein peak on the disc electrophoresis.

Estimation of molecular weight

The molecular weight of PLase A was estimated to be about 26,800 by gel filtration with Sephadex G-200 (Fig. 2). It was shown by sodium dodecyl sulfate disc electrophoresis that PLase A consisted of one polypeptide chain.

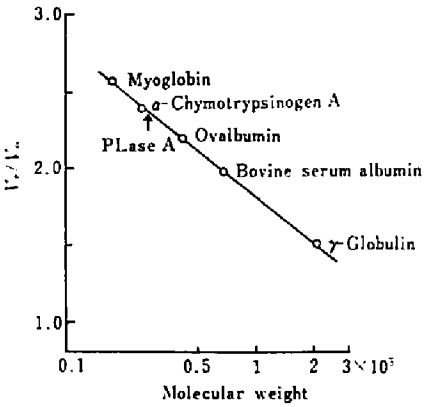

FIG. 2. Estimation of Molecular Weight of PLase A by Gel Filtration.

Chromatography was carried out on a Sephadex G-200 column (1.5 × 92 cm) with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl, after the column had been equilibrated with the same buffer, and 2 ml fractions were collected. V_e, the elution volume; V_0, the void volume.

Effects of pH on PLase A and LPLase activities

As shown in Fig. 3, PLase A and LPLase activities had their pH optimums between 4.0 and 4.5, and were most stable in the pH range of 6.0 to 8.0. Both enzyme activities were unstable on the acidic side.

Thermal stability

Both PLase A and LPLase activities were rapidly lost at 45°C, pH 7.0 (Fig. 4).

Positional specificity of PLase A

The products of the enzyme reaction which had proceeded to about 10% hydrolysis of the substrate were analyzed by using the purified PLase A and the crude enzyme preparation

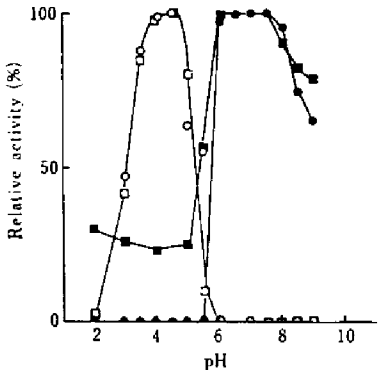

FIG. 3. Effects of pH on PLase A and LPLase Activities.

(a) Optimal pH: the enzyme activities were measured by the standard assay method in the following buffers: 0.1 M sodium acetate-HCl buffer (pH 2.0~3.0), 0.1 M acetate buffer (pH 3.5~5.0), 0.2 M Tris-malate-NaOH buffer (pH 5.5~8.0) and 0.05 M Tris-HCl buffer (pH 8.5~9.0).
○—○, PLase A activity; □—□, LPLase activity.
(b) pH stability: the enzyme solution was incubated at each pH value for 45 hr at 4°C, and the remaining activities were assayed. Buffers used were the same as described above.
●—●, PLase A activity; ■—■, LPLase activity.

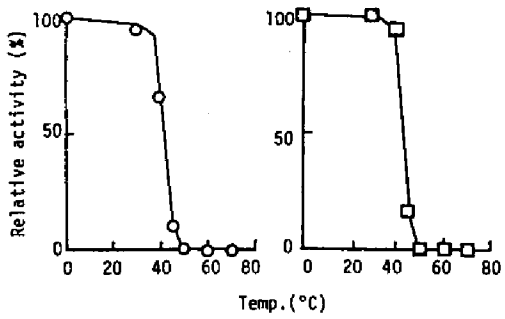

FIG. 4. Thermal Stability of PLase A and LPLase Activities.
The enzyme solution was incubated at various temperatures for 15 min at pH 7.0, and the remaining activities were assayed.
○—○, PLase A activity; □—□, LPLase activity.

Table II. Positional Specificity of PLase A Activity

The procedure used was described in Materials and Methods.

Enzyme	Hydrolysis (%)	1-Monoglyceride (%)	2-Monoglyceride (%)
Purified PLase A	12.6	3.6	96.4
Crude enzyme	10.9	24.1	75.9

(Table II). When the purified PLase A was used, 96.4% of monoglycerides were found to be 2-monoglycerides. This result indicated that the type of the purified PLase A was A_1. On the other hand, when the crude enzyme preparation was used, the proportions of 1- and 2-monoglycerides were found to be 24.1% and 75.9%, respectively. From this result, it was estimated that PLase A_2 and PLase A_1 activities existed at a ratio of about 1 to 3 in the culture filtrate of *C. centrifugum*.

Positional specificity of LPLase activity

The positional specificity of LPLase activity was decided by comparing 1-acyl-LPC and 2-acyl-LPC as to their effectiveness as substrate for LPLase activity. As shown in Table III, the purified enzyme released 565 nmoles of FFA for 10 min from 1-acyl-LPC, but only 30 nmoles of FFA from 2-acyl-LPC under the same assay conditions. The ratio between 1-acyl-LPC and 2-acyl-LPC in relative

Table III. Positional Specificity of LPLase Activity

The enzyme activity was measured by the standard assay method with 1-acyl-LPC or 2-acyl-LPC as substrate.

Substrate	Free fatty acid released (nmole, (%))
1-Acyl-LPC	565 (100)
2-Acyl-LPC	30 (5)

activity of LPLase was 100 : 5. From this result, the type of LPLase activity was decided to be LPLase 1.

Effects of reagents

Neither PLase A nor LPLase activity was affected by EDTA and SH reagents, but they were strongly inhibited by *p*-diazobenzenesulfonic acid and *N*-bromosuccinimide at 1 mM concentration (Table IV).

Table IV. Effects of Various Reagents on PLase A and LPLase Activities

The enzyme was incubated with each reagent in 0.05 M phosphate buffer, pH 7.2, at 30°C for 30 min, and the residual activity was measured by the standard assay method according to Duncombe,[17] except that PLase A activity was measured by using 1 mM PC instead of LPC and adding 10% 1-propanol.

Reagent	Conc. (mM)	Activity (%)	
		PLase A	LPLase
None	—	100	100
EDTA	10	97	97
N-Ethylmaleimide	10	87	100
Monoiodoacetate	10	94	99
Iodoacetamide	10	98	95
p-Diazobenzenesulfonic acid	1	20	0
	10	5	0
N-Bromosuccinimide	1	0	0

Effects of urea

Both PLase A and LPLase activities were inactivated at the same rate in 0.05 M Tris-HCl buffer, pH 7.0, containing 2 M urea at 30°C (Fig. 5).

Effects of organic solvents and detergents

The enzyme activity was measured by adding the enzyme to a mixture of substrate and various organic solvents or detergents (Table V). PLase A activity was generally activated by organic solvents except methanol. Ether and 1-propanol activated PLase A

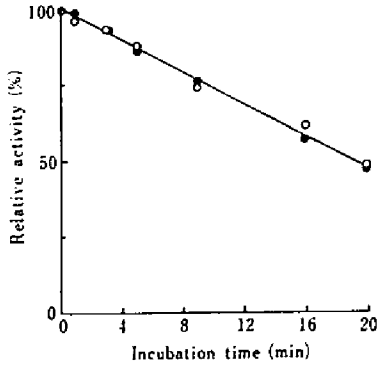

FIG. 5. Effects of Urea on PLase A and LPLase Activities.

The enzyme was incubated for various lengths of time in 0.05 M Tris-HCl buffer, pH 7.0, containing 2 M urea at 30°C, and the residual activities were measured by the standard assay method.
○—○, PLase A activity; ●—●, LPLase activity.

Table V. Effects of Organic Solvents and Detergents on PLase A and LPLase Activities

The enzyme activities were measured by the standard assay method except that one of the listed organic solvents or detergents at the indicated concentration was added before the reaction.

Reagent	Conc. (%)	Activity (%)	
		PLase A	LPLase
None	—	100	100
Ether	10	397	21
Methanol	10	45	97
Ethanol	10	108	84
1-Propanol	10	630	83
2-Propanol	10	129	100
Acetone	10	137	81
Sodium dodecyl sulfate	0.1	0	0
Cetyltrimethylammonium bromide	0.1	99	
Triton X-100	0.1	1100	101
	0.2	4250	—
	0.4	5350	84
Tween 80	0.1	—	92
	0.4	—	51

activity about 4- and 6-fold, respectively, at a concentration of 10%. LPLase activity was inhibited by ether, but it was not significantly affected by other organic solvents. Both enzyme activities were completely inhibited by 0.1% sodium dodecyl sulfate. An anionic detergent, cetyltrimethylammonium bromide, did not affect PLase A activity, but substantially inhibited all LPLase activity. Triton X-100 little affected LPLase activity, but it strongly activated PLase A activity from 11- to 53.5-fold at concentrations of 0.1 to 0.4%.

Effects of metal ions

PLase A activity was almost completely inhibited by Fe^{2+}, Fe^{3+} and Al^{3+}, when Triton X-100 was not included in the assay medium (Table VI). But 94, 28 and 72% of the relative activities of PLase A affected by Fe^{2+}, Fe^{3+} and Al^{3+}, respectively, were restored by the addition of 0.2% Triton X-100. On the other hand,

TABLE VI. EFFECTS OF METAL IONS ON PLASE A AND LPLASE ACTIVITIES

The enzyme activities were measured by the standard assay method except that 10 mM of one of the indicated reagents was added before the reaction. The activity is expressed in percentage of the activity level in the absence of metal salts. Fe^{2+} was used in the sulfate form, and the other metal ions were used in the chloride form.

Metal ion (10 mM)	Activity (%)		
	PLase A		LPLase
	without Triton	with Triton	
None	100	100	100
Ca^{2+}	97	96	84
Hg^{2+}	83	99	46
Fe^{2+}	0	94	80
Fe^{3+}	7	28	4
Al^{3+}	0	72	41

LPLase activity was inhibited by Hg^{2+}, Fe^{3+} and Al^{3+}.

Mode of inhibition of LPLase activity by Fe^{3+}

As shown in Fig. 6, the Lineweaver-Burk plots were parallel, indicating that LPLase activity was inhibited by Fe^{3+} in an uncompetitive mode. It was observed that, when the substrate concentration increased by more than 2 mM, the inhibition of LPLase activity by 0.2 mM Fe^{3+} decreased. Irrespective of the existence or nonexistence of Fe^{3+}, the Lineweaver-Burk plots broke upward at 0.36 mM concentration of the substrate. The apparent Michaelis constant of LPLase activity was calculated to be 0.2 mM.

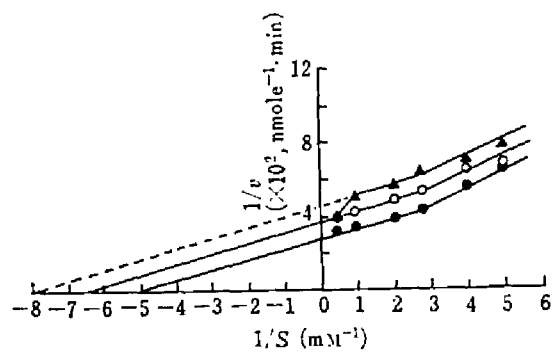

Fig. 6. Effects of Fe^{3+} Concentration on LPLase Activity.

The enzyme activity was measured by the standard assay method except that varying concentrations of Fe^{3+} were added before the reaction.

●—●, without Fe^{3+}; ○—○, 0.15 mM Fe^{3+};
▲—▲, 0.2 mM Fe^{3+}.

Substrate specificity of PLase A

PLase A showed a wide substrate specificity, and hydrolyzed PC, PE, PI, PG, CL and PS in this order, but did not attacked triolein (Table VII).

Table VII. Substrate Specificity of PLase A

The enzyme activity was measured by the standard assay method with each of the indicated substrate.

Substrate	Fatty acyl ester decreased/10 min (μmoles)
PC	0.70
PE	0.61
PI	0.27
PG	0.23
CL	0.21
PS	0.10
Triolein	0.00

Discussion

PLases A were classified into two types according to their positional specificities. The enzymes that catalyze the hydrolysis of the fatty acids at position 1 and 2 of the glycerol moiety of phospholipids are called PLase A_1 and PLase A_2, respectively.[21] Hitherto PLase A_2 has been better known than PLase A_1. PLase A_2, especially from snake venoms, has been used as reagent for the preparation of 1-acyllysophospholipids, the proof of the distribution of fatty acids on phospholipids, and the decomposition of phospholipids of red cell membranes. On the other hand, the existence of PLase A_1 was not recognized before 1960. In recent years, however, highly purified enzymes were reported from two species of bacteria[37-38] and beef pancreas.[39] As described in Results, the positional specificity of the purified PLase A of

C. centrifugum in the present study was determined as that of PLase A_1.

The facts that (i) the ratio of PLase A activity to LPLase activity was constant at every purification step after the first DEAE-Sephadex column chromatography, (ii) they were similar to each other in optimum pH, pH stability and heat stability and (iii) protein modifying reagents and urea gave the same effects suggest that the same enzyme protein shows both PLase A_1 and LPLase 1 activities. The different effects of organic solvents, detergents and metal ions on the two enzyme activities, on the other hand, may be ascribed to the characters of these substances; namely, they not only bind to the enzyme protein but also change the dispersion state of the substrate.

The molecular weight of PLase A_1 from *C. centrifugum* was similar to those from *E. coli*[37] and *Bacillus megaterium*.[38] PLase A_1 from *C. centrifugum* hydrolyzed 1-acyl-LPC and other phospholipids as that from *E. coli*[37] did, but was inhibited by sodium dodecyl sulfate, and did not require Ca^{2+} for its activity.

As was PLase A_1 from rat brain,[40] that from *C. centrifugum* was activated by Triton X-100.

The Lineweaver-Burk plots of LPLase activity broke upward at 0.36 mM concentration of the substrate (Fig. 6). The critical micelle concentration of LPC in 0.1 M acetate buffer, pH 4.0, exists between 0.25 and 0.40 mM (unpublished data). So it was

presumed that the micellar form was a better substrate for LPLase activity than the monomolecular form. When the substrate concentration increased by more than 2 mM, the inhibition of LPLase activity by 0.2 mM Fe^{3+} decreased. This result suggested that, when substrate concentration increased, Fe^{3+} bound to substrate increased and free Fe^{3+} decreased, resulting in a reduced inhibition by Fe^{3+}.

As PLase A_1 from *C. centrifugum* did not hydrolyze triolein, nor that from *E. coli*[37] either, this PLase A_1 activity presumably did not originate from a nonspecific lipase.

C. centrifugum is the first mold in which PLase A_1 was found. It was the first time that PLase A_1 was found in a plant pathogen. The significance of the existence of PLase A_1 in this mold was considered to lie in the destruction of the cell membrane of plant tissues and the decomposition of phospholipids as a source of nutrients for the mold.

Since PLase A_1 from *C. centrifugum* is an extracellular enzyme and produced abundantly, this enzyme is expected to become available for wide use as a reagent for the determination of the fatty acid composition at position 1 of phospholipids, as well as snake venom PLase A_2 at position 2.

CHAPTER III

PARTIAL PURIFICATION AND PROPERTIES OF PHOSPHOLIPASE B PRODUCED BY *CORTICIUM CENTRIFUGUM*

As described in Chapter II, the presence of PLase A_1 was found in the culture broth of the plant pathogenic mold, *C. centrifugum*. PLase A_1 produces a cytotoxic substance, lysophospholipids, by attacking diacylglycerophospholipids. From the analogy with a snake venom PLase A_2, it was presumed that PLase A_1 of this mold could become the cause of plant cell death by inducing the lysis of cell membranes. In order to determine whether this effectively toxic action against plant cells is a well supposable hypothesis or not, it is needed to study PLase A_1 in comparison with the all PLases produced by *C. centrifugum*. Hasegawa *et al.*[9] reported the presence of PLases A and B and LPLase in *C. centrifugum*. However, their detailed properties were not investigated as yet. In this time, another lecithin acyl-hydrolase active fraction that is different from PLase A_1 was found under the assay condition without a detergent. The heat treatment examination and analysis of the reaction products demonstrated that this lecithin acyl-hydrolase was PLase B, which hydrolyzed both of fatty acyl ester groups of diacylglycerophospholipids.

In this Chapter, the partial purification and properties of PLase B produced by *C. centrifugum* are described.

The following nomenclature is used in this Chapter in

accordance with that proposed by Ansell and Hawthorne.[41] The term PLase A denotes the enzyme that hydrolyzes one of the acyl ester bonds of the phospholipid molecule (EC 3.1.1.4) and PLase B denotes the enzyme or enzyme system that hydrolyzes both the acyl ester bonds of the phospholipid molecule. The term LPLase denotes the enzyme that hydrolyzes the acyl ester bond of lysophospholipid molecule (EC 3.1.1.5). The term lecithin acyl-hydrolase was used in the cases in which the activity remained unidentified as either PLase A or B.

Materials and Methods

Enzyme. As described in Chapter II, *C. centrifugum* IAM 9028 was cultured in a medium containing 4% of heat-treated brewer's yeast as a main nutritious source, at 28°C for 72 hr. The proteins were precipitated by saturation with ammonium sulfate, and its solution was desalted by Sephadex G-25 column chromatography. The desalted solution was purified by DEAE-Sephadex and hydroxylapatite column chromatography.

Substrate. Various substrates used in this experiment were prepared as described in Chapter II.

Enzyme assay. Lecithin acyl-hydrolase activity was assayed as follows: the incubation mixture contained 0.5 ml of ultrasonic dispersion of PC (2 μmoles), which was sonicated with an ultrasonic oscillator (Branson Sonifier), 10 kHz, for 2 min in 0.1 M acetate

buffer, pH 5.0, 0.4 ml of 0.1 M acetate buffer, pH 5.0, and 0.1 ml of enzyme solution in a total volume of 1 ml. Incubation was carried out at 30°C for 15 min. The reaction was stopped by the addition of 2.5 ml of methanol and 1.25 ml of chloroform, immediately followed by chilling at 0°C. According to the method of Bligh and Dyer,[25] 1.25 ml of chloroform and 1.25 ml of water were added with shaking at each time. The tube was centrifuged for 5 min, and a 1 ml aliquot of the upper water-methanol phase was taken to a test tube. After evaporated off, water-soluble organic phosphate, which was based on GPC, was measured by the method of Bartlett.[42] In the experiment of the time course of lecithin acyl-hydrolase, a 2 ml aliquot of the chloroform phase was taken, and the decrease in fatty acyl ester group was measured by the method of Antonis.[26]

LPLase activity was assayed as described in Chapter II.

PLases C and D activities were assayed as follows: the incubation mixture contained 0.5 ml of ultrasonic dispersion of PC (2 μmoles) which was sonicated with an ultrasonic oscillator in 0.1 M acetate buffer, pH 4.0, containing 0.4% Triton X-100, 0.4 ml of 0.1 M acetate buffer, pH 4.0, and 5 μg of the crude enzyme, a lyophilized preparation of the desalted solution of the precipitate from the culture filtrate by saturation with ammonium sulfate, in a total volume of 1 ml. Incubation was carried out at 30°C for 20 min. The reaction was stopped by the addition of 2.5 ml of metha-

nol and 1.25 ml of chloroform. Further 1.25 ml of chloroform and 1.25 ml of water were added in accordance with the method of Bligh and Dyer.[25] Diglyceride and phosphorylcholine, the reaction products of PLase C activity, were detected by TLC. Choline, one of the reaction products of PLase D activity, was also detected by TLC.

One unit each of lecithin acyl-hydrolase and LPLase activities was defined as the amount of the corresponding enzyme which liberated 1 μmole each of phosphorus or FFA per min under the standard assay conditions.

Thin-layer chromatography. FFA, LPC and diglycerides in the chloroform phase of the method of Bligh and Dyer[25] were analyzed by TLC, using Silica Gel G plates (Merck) with chloroform-methanol-water (80:35:5, v/v/v) as solvent.[9] Spots were visualized by iodine vapor. GPC, phosphorylcholine and choline, which were extracted in the water-methanol phase of the method of Bligh and Dyer,[25] were analyzed by TLC using Silica Gel G plates with phenol-saturated water as solvent.[9] Spots were visualized by iodine vapor and phosphate spraying reagent.

Isoelectric focusing and determination of protein concentration and molecular weight. All these procedures are the same as that described in Chapter II.

Results

Enzyme purification procedure

Step 1. First DEAE-Sephadex column chromatography. The desalted solution was applied to a DEAE-Sephadex A-25 column (Fig. 1).

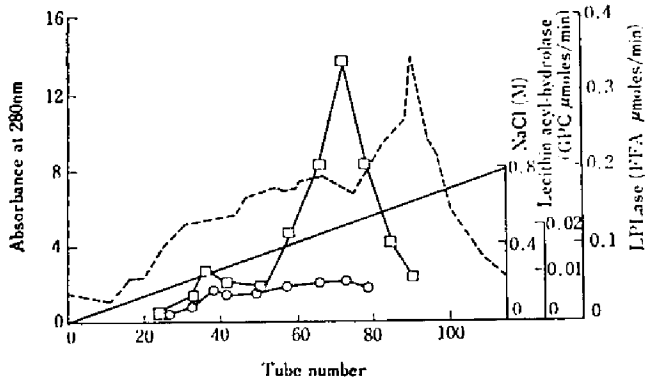

Fig. 1. First DEAE-Sephadex Column Chromatography of Lecithin Acyl-hydrolase and LPLase from *C. centrifugum*.

The desalted solution was applied to a DEAE-Sephadex A-25 column (2.6 × 37.5 cm), which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2. After washing inactive proteins with the same buffer, the enzymes were eluted with a linear gradient of NaCl concentration from zero to 0.8 M in the same buffer at a rate of 40 ml/hr, and 10 ml fractions were collected.

- - -, absorbance at 280 nm; ○——○, Lecithin acyl-hydrolase activity; □——□, LPLase activity; ——, NaCl concentration.

Lecithin acyl-hydrolase activity was eluted with the NaCl concentrations of 0.25 and 0.45 M. The first active fractions, tube Nos. 34-46, were combined, concentrated and then dialyzed against 0.05 M Tris-HCl buffer, pH 7.2, with a collodion-bag.*

Step 2. Second DEAE-Sephadex column chromatography. The dialyzed solution obtained above was applied to a DEAE-Sephadex A-25 column (1.6 x 30 cm), which was equilibrated with 0.05 M Tris-

HCl buffer, pH 7.2. After eluting inactive proteins with the same buffer, the enzyme was eluted with a linear gradient of NaCl concentration from zero to 0.45 M in the same buffer at a rate of 30 ml/hr, and 5 ml fractions were collected. The combined active fractions were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.1, with a collodion-bag.

Step 3. Hydroxylapatite column chromatography. The dialyzed solution was applied to a hydroxylapatite column (2.6 x 10 cm), which was composed of hydroxylapatite and cellulose (1:1, v/v), and was equilibrated with 10 mM phosphate buffer, pH 7.1. Elution was carried out at a rate of 30 ml/hr with a stepwise increase in buffer concentration (10, 50 and 100 mM), and 5 ml fractions were collected. The combined active fractions, which were eluted with 50 mM phosphate buffer, were concentrated and dialyzed against 10 mM phosphate buffer, pH 7.1, with a collodion-bag. The dialyzed solution was preserved at -20°C.

The result of purification by the above procedures are summarized in Table I. Lecithin acyl-hydrolase and LPLase showed

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* The second lecithin acyl-hydrolase activity, tube Nos. 60-80, seemed to be an analog of the first lecithin acyl-hydrolase activity, tube Nos. 34-46, because, on a rechromatography of the second one, the most of the lecithin acyl-hydrolase activity was eluted with the NaCl concentration of 0.25 M. A further purification was not carried out on the second one. The properties of LPLase activity eluted with the NaCl concentration of 0.45 M have been described in Chapter II, and this enzyme was not dealt with in this Chapter.

Table I. Summary of Purification of Lecithin Acyl-hydrolase and LPLase

Procedure	Protein	Activity (units)		Specific activity (units/mg)		LPLase / Lecithin acyl-hydrolase
	(mg)	Lecithin acyl-hydrolase	LPLase	Lecithin acyl-hydrolase	LPLase	
$(NH_4)_2SO_4$ ppt. (100% satn.)	21094	4047	37200	0.19	1.76	9.3
1st DEAE-Sephadex	766	273	1267	0.36	1.65	4.6
2nd DEAE-Sephadex	151	101	643	0.67	4.26	6.4
Hydroxylapatite	11	9	238	0.87	22.60	26.0

4.6- and 12.8-fold increase in specific activity, respectively. The ratio of both enzyme activities showed different values at every purification step. This difference demonstrates that both enzyme activities originate in different enzymes.

Homogeneity of lecithin acyl-hydrolase activity of the enzyme preparation purified by hydroxylapatite

The hydroxylapatite-purified enzyme preparation was applied to a Sephadex G-200 column (Fig. 2). This enzyme preparation contained one component of lecithin acyl-hydrolase activity, tube Nos. 42-50, and two components of LPLase activity, as a peak, tube Nos. 34-40, and a shoulder, tube Nos. 42-50. The isoelectric focusing also showed that lecithin acyl-hydrolase fraction was composed of a single active component. The isoelectric point of lecithin acyl-hydrolase was found to be 4.04.

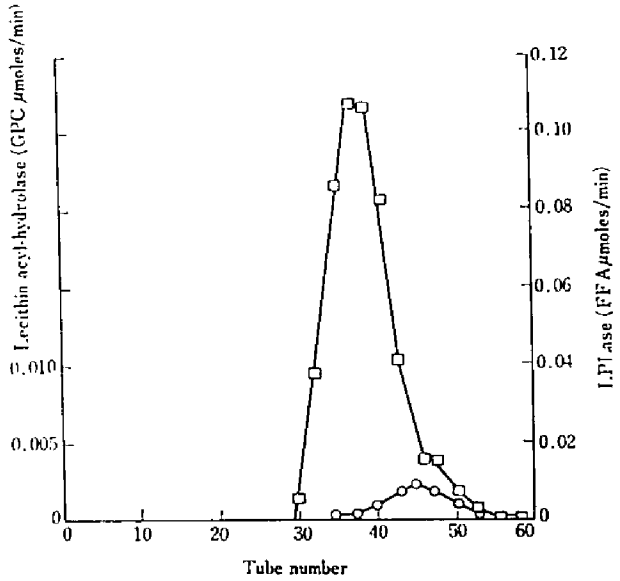


Fig. 2. Sephadex G-200 Column Chromatography of the Hydroxylapatite-Purified Enzyme Preparation.

The enzyme solution was applied to a Sephadex G-200 column (1.5 × 92 cm), which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl. The elution was carried out with the same buffer, and 2 ml fractions were collected.

○—○, Lecithin acyl-hydrolase activity; □—□, LPLase activity.

Determination of the type of reaction of lecithin acyl-hydrolase

The time course of lecithin acyl-hydrolase activity is shown in Fig. 3. The hydrolysis reaction showed almost a linear rate up to about 20 min incubation. The amount of GPC formed coincided stoichiometrically with the amount of ester hydrolyzed, at the ratio of 1 μmole of GPC for 2 μeq of decrease in ester. This result seems to demonstrate that lecithin acyl-hydrolase activity was exhibited by PLase B. In fact, this assumption was also ascertained by TLC, that is, the reaction products were only FFA and GPC

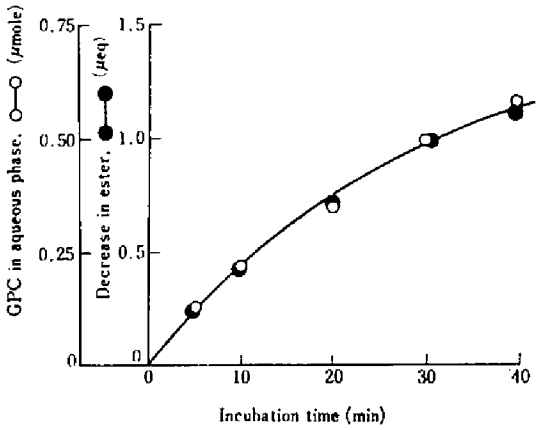


Fig. 3. Time Course of Lecithin Acyl-hydrolase Activity.
Incubation mixtures contained 0.5 ml of ultrasonic dispersion of PC (2 μmoles), 0.4 ml of 0.1 M acetate buffer, pH 5.0, and 0.1 ml of enzyme solution in a total volume of 1 ml. Incubation was carried out at 30°C. Hydrolysis was measured by the decrease in fatty acyl ester groups and the increase in GPC.

under the standard assay conditions. However, there was another possibility that PLase B activity was shown apparently by the successive actions of PLase A and LPLase, because the partially purified enzyme preparation contained a strong LPLase activity. But LPC, the product of PLase A activity, was not detected by TLC in the experiment using the lecithin acyl-hydrolase active fraction, tube Nos. 42-50 in Fig. 2, which had been removed about 90% of LPLase activity from the hydroxylapatite-purified enzyme preparation.

As shown in Table II, when the hydroxylapatite-purified enzyme preparation was treated at 45°C for 15 min at pH 7.0, LPLase activity was almost completely inactivated. On the other hand, 69% of lecithin acyl-hydrolase activity still remained. Over 50°C,

Table II. Thermal Stability of Lecithin Acyl-hydrolase and LPLase Activities.

The enzyme solution was incubated at various temperatures for 15 min at pH 7.0, and the remaining activity was assayed.

Temperature (°C)	Relative Activity (%)	
	Lecithin acyl-hydrolase	LPLase
0	100	100
30	106	100
40	96	43
45	69	3
50	30	0
60	22	0
70	16	0

LPLase activity was completely inactivated, while lecithin acyl-hydrolase activity retained 16% of its activity even at 70°C. If lecithin acyl-hydrolase activity is the result of the successive actions of PLase A and LPLase activities, the enzyme preparation which was inactivated LPLase activity by heat treatment but still retained the residual PLase A activity should produce LPC as an intermediate product, when it attacks PC. In the heat treatment experiment, however, LPC was not detected by TLC. These results demonstrate that the lecithin acyl-hydrolase activity of the hydroxylapatite-purified enzyme preparation is due to PLase B. In the following description, lecithin acyl-hydrolase activity was denoted as PLase B activity. The following examinations about the properties of PLase B activity were carried out by using this hydroxylapatite-purified enzyme preparation. One unit of PLase B activity was defined as same as that of lecithin acyl-hydrolase

activity described above.

Estimation of the molecular weight

The molecular weight of PLase B was estimated to be about 48,000 by the gel filtration as shown in Fig. 4.

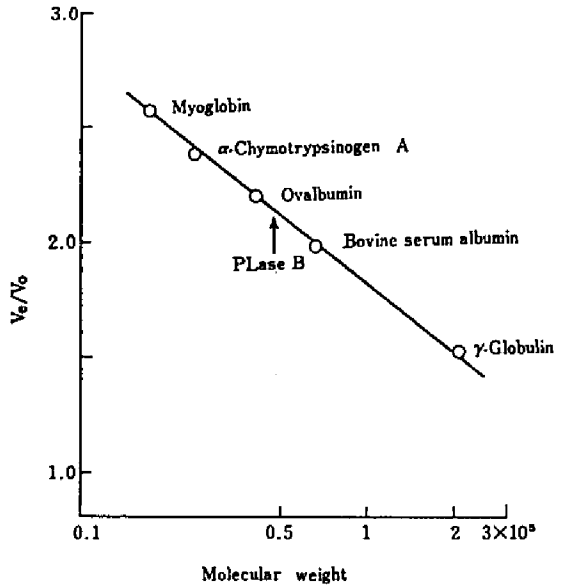


Fig. 4. Estimation of Molecular Weight of PLase B by Gel Filtration.

Chromatography was carried out on a Sephadex G-200 column (1.5 x 92 cm) with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl, after the column had been equilibrated with the same buffer, and 2 ml fractions were collected.
Ve, the elution volume; Vo, the void volume.

Optimal pH

As shown in Fig. 5, the optimal pH of PLase B activity was found to be 5.5.

pH stability

As shown in Fig. 6, PLase B was most stable at pH 5.5-8.0, and

more stable on the acidic side than on the alkaline side.

Effects of reagents

The effects of various reagents on PLase B activity are shown in Table III. EDTA and SH reagents did not affect PLase B activity. But, *p*-diazobenzenesulfonic acid and *N*-bromosuccinimide inhibited the enzyme activity at 1 mM.

Effects of organic solvents and detergents

As shown in Table IV, PLase B activity was inhibited by various organic solvents, and was almost completely inhibited by 0.1% sodium dodecyl sulfate, and generally received inhibitory effects by nonionic and cationic detergents.

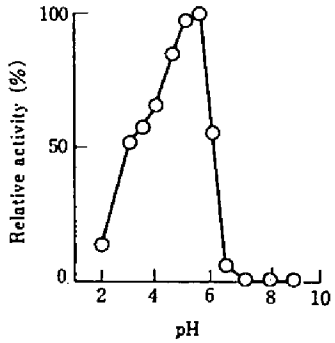


Fig. 5. Optimal pH of PLase B Activity.

The enzyme activity was measured by the standard assay method in the following buffers: 0.1 M sodium acetate–HCl buffer (pH 2.0 - 3.0), 0.1 M acetate buffer (pH 3.5 - 5.0), 0.2 M Tris-malate–NaOH buffer (pH 5.5 - 8.0) and 0.05 M Tris-HCl buffer (pH 8.5 - 9.0).

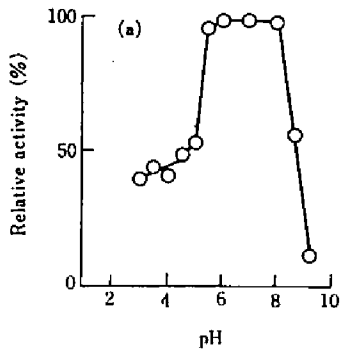


Fig. 6. pH Stability of PLase B Activity.

The enzyme solution was incubated at each pH value for 45 hr at 4°C, and the remaining activities were assayed. Buffers used were 0.1 M sodium acetate–HCl buffer (pH 2.0 - 3.0), 0.1 M acetate buffer (pH 3.5 - 5.0), 0.2 M Tris-malate–NaOH buffer (pH 5.5 - 8.0) and 0.05 M Tris-HCl buffer (pH 8.5 - 9.0).

Table III. Effects of Various Reagents on PLase B Activity

The enzyme was incubated with each reagent in 0.05 M phosphate buffer, pH 7.2, at 30°C for 30 min, and the residual activity was measured by the standard assay method.

Reagent	Conc. (mM)	Activity (%)
None	-	100
EDTA	10	100
N-Ethylmaleimide	10	100
Monoiodoacetic acid	10	102
Iodoacetamide	10	102
p-Diazobenzenesulfonic acid	1	45
	10	13
N-Bromosuccinimide	1	0

Table IV. Effects of Organic Solvents and Detergents on PLase B Activity

The enzyme activity was measured by the standard assay method except that one of the listed organic solvents and detergents at the indicated concentration was added before the reaction.

Reagent	Conc. (%)	Activity (%)
None	-	100
Ether	10	29
Methanol	10	56
Ethanol	10	41
1-Propanol	10	6
2-Propanol	10	41
Acetone	10	47
Sodium dodecyl sulfate	0.1	6
	0.4	0
Triton X-100	0.1	85
Tween 80	0.1	49
	0.4	23
Tween 20	0.1	49
	0.4	68

Effects of metal ions

Though Ba^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} slightly stimulated PLase B activity, Fe^{2+}, Fe^{3+} and Al^{3+} substantially inhibited PLase B activity at 10 mM concentration (Table V).

Table V. Effects of Metal Ions on PLase B Activity

The enzyme activity was measured by the standard assay method except that 10 mM of one of the indicated reagents was added before the reaction. The activity is expressed in percentage of the activity level in the absence of metal salts. Fe^{2+} was used as the sulfate form, and the other metal ions were used as the chloride form.

Metal ion (10 mM)	Activity (%)
None	100
Li^{+}	98
Ba^{2+}	110
Ca^{2+}	108
Mg^{2+}	103
Mn^{2+}	115
Fe^{2+}	2
Cu^{2+}	54
Zn^{2+}	88
Cd^{2+}	94
Hg^{2+}	76
Fe^{3+}	2
Al^{3+}	4

Mode of inhibition of PLase B activity by metal ions

The modes of inhibition of PLase B activity by Fe^{2+}, Fe^{3+} and Al^{3+} were investigated by the Lineweaver-Burk plots (Fig. 7).

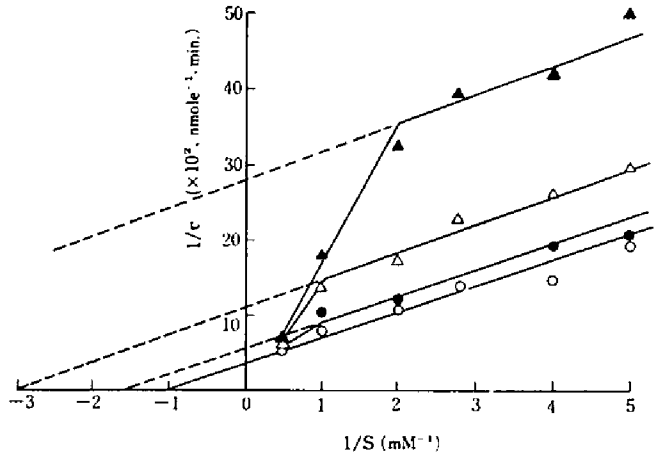


Fig. 7. Effects of Various Metal Ions on PLase B Activity at Various Concentrations of PC.

The enzyme activity was measured by the standard assay method except that one of the indicated metal ions was added before the reaction.

○——○, without metal ion; ●——●, 0.004 mM Fe^{3+};
△——△, 0.02 mM Al^{3+}; ▲——▲, 0.003 mM Fe^{2+}.

Since the plots in the presence of each Fe^{2+}, Fe^{3+} and Al^{3+} were parallel, it was suggested that these metal ions inhibited PLase B activity in an apparent uncompetitive mode. And, it was observed that inhibition by metal ions decreased at over 1 mM substrate concentration. The apparent Michaelis constant of PLase B activity was calculated to be 1 mM.

Substrate specificity of PLase B

As shown in Table VI, PLase B showed a wide substrate specificity about the various phospholipids contained in plant tissues. PC, PI and PG were hydrolyzed well, and PE, CL and PS were also attacked.

Table VI. Substrate Specificity of PLase B

The enzyme activity was measured by the standard assay method using each of the indicated substrates except that 0.1% bovine serum albumin was added before the reaction.

Substrate	Free fatty acid released/10 min (μmoles)
PC	0.20
PI	0.18
PG	0.17
PE	0.05
CL	0.02
PS	0.02

Discussion

The optimal pH of PLase B from *Corticium centrifugum* was found to be 5.5 in the present study, whereas Tseng *et al.*[7] reported that of PLase B from *Sclerotium rolfsii*, another name of *Corticium centrifugum*, to be 4.5. In the experiment by Tseng *et al.*,[7] PLase B was reported as an only PLase in *S. rolfsii*. In the present study, however, PLase A_1, PLase B and LPLase were found in *C. centrifugum*. These differences may be due to the differences in the culture conditions, assay conditions and the strains used. On the other hand, as well as Tseng *et al.*[7] PLases C and D activities were not detected in the culture filtrate of this mold.

PLase B from *Penicillium notatum*[44-45] was stimulated by sodium dodecyl sulfate. But, PLase B from *C. centrifugum* was inhibited by this detergent as well as that from *Streptomyces hiroshimensis*.[46]

PLase B from *S. hiroshimensis*[46] was activated by Ca^{2+} and Ba^{2+}, and was inhibited by Zn^{2+}, Fe^{3+} and Al^{3+}. This result is similar to that of PLase B from *C. centrifugum* except the effect of Zn^{2+}. On the other hand, PLase B from *P. notatum*[47] was inhibited by Fe^{2+} and Fe^{3+}. It seems that Fe^{2+} and Fe^{3+} are generally inhibitory to PLase B activity. The difference of the kind of inhibitory metal ions against these enzymes may be based on the difference of the states of binding sites of metal ions on enzyme proteins. The inhibition by metal ions decreased at over 1 mM

substrate concentration as shown in Fig. 7. This result suggested that when substrate concentration increased, metal ions bound to substrate increased and free metal ions decreased, resulting in a reduced inhibition by metal ions.

CHAPTER IV

PURIFICATION AND PROPERTIES OF LYSOPHOSPHOLIPASE PRODUCED BY *CORTICIUM CENTRIFUGUM*

It has previously been demonstrated that PLases A and B and LPLase were present in the cell wall lytic enzyme preparation, which was prepared from the culture broth of *Corticium centrifugum*.[9] To make clear the properties of and relation among these enzymes, purification of LPLase was attempted from the culture broth of this mold.

In this Chapter, the purification and properties of LPLase produced by *C. centrifugum* are described.

The crude enzyme was prepared from the culture broth by the following two different methods. In Section IV-1, the results obtained with the enzyme proteins which were collected by the fractionation with ammonium sulfate after concentrating the culture filtrate by ultrafiltration are described. On the other hand, in Section IV-2, the results obtained with the enzyme proteins which were directly collected from the culture filtrate by the fractionation with ammonium sulfate without ultrafiltration are described.

It will be shown in Section IV-1 that the purified LPLase has a high substrate specificity and doesn't attack PC and monoglycerides. In Section IV-2, the determination of the reaction type of LPLase activity will be described.

Section IV-1. Purification and Properties of Lysophospholipase Produced by *C. centrifugum*: Purification with Ultrafiltration and Substrate Specificity

Materials and Methods

Enzyme. As described in Chapter II, *C. centrifugum* IAM 9028 was cultured in a medium containing 4% of heat-treated brewer's yeast as a main nutritious source, at 26-28°C for 67-93 hr. The culture broth was neutralized by 2N NaOH solution, and the mycelia were eliminated by continuous centrifugation. The supernatant was filtered through two layers of cheese cloth and pulp layer, followed by ultrafiltration. By this procedure, 40 liters of filtrate was concentrated to about 4 liters. The precipitate from the concentrated culture filtrate obtained by saturation with ammonium sulfate was collected and dialyzed against water for only 4 hr (because of the damage of the cellulose tubing by a cell wall lytic activity in the culture filtrate), followed by lyophilization. The purification of this crude enzyme preparation was done twice. The respective purified enzyme preparations were numbered I and II, of which specific activities were 68.0 and 50.9 units/mg, respectively. On unit of activity was defined as the amount of enzyme which liberated 1 μmole of fatty acid per min.

Substrate. PC was prepared from egg yolk by the method of Pangborn,[48] and was purified by silicic acid column chromatogra-

phy. LPC was prepared by the action of *Vipera russelli* PLase A (Sigma Chem. Co.) or *Crotalus adamanteus* PLase A (Worthington Biochem. Co.) on PC by the procedure of Hanahan,[23] and purified by silicic acid column chromatography. [U-^{14}C]-1-Acyl-LPC was prepared by the action of *Vipera russelli* PLase A on [U-^{14}C]-PC (New England Nuclear Co.) which was diluted with unlabeled PC from egg yolk previously. [1-^{14}C]-Palmitoyl-LPC was prepared by the action of *Crotalus adamanteus* PLase A (Sigma Chem. Co.) on L-α-dipalmitoyl-[1-^{14}C]-PC (Applied Science Laboratories Inc.) which was diluted with unlabeled dipalmitoyl-PC (Serdary Research Laboratories) previously. [1-^{14}C]-Oleoyl-LPC was prepared by the same method as described above from L-α-dioleoyl-[1-^{14}C]-PC (Applied Science Laboratories Inc.) which was diluted with unlabeled dioleoyl-PC (Serdary Research Laboratories). These LPCs were purified by silicic acid column chromatography. The specific radioactivities of [U-^{14}C]-1-acyl-LPC, [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC were 30, 45 and 95 cpm/mμmole LPC, respectively.

Enzyme assay. LPLase activity, when unlabeled LPC was used as substrate, was assayed as described in Chapter II. In some cases in which enzyme preparation I was used, incubation was carried out in 0.1 M acetate buffer, pH 3.6, at 45°C.

For assay of lecithin acyl-hydrolase, 0.5 ml of ultrasonic dispersion of PC (2 μmoles), which was sonicated with an ultrasonic oscillator (Kaijo Denki Model 425IS), 10 kHz, for 1 min, was used

as substrate in place of aqueous solution of LPC. Other conditions and procedures were the same as above.

When labeled LPC was used as substrate, the reaction mixture contained LPC (1 μmole) which was pipetted in the reaction tube as methanol solution and evaporated, 0.9 ml of 0.1 M acetate buffer, pH 4.0, and 0.1 ml of enzyme solution in a total volume of 1 ml. Enzyme reaction was carried out at 30°C for 8 min. The reaction was stopped by addition of 2.5 volumes of methanol and by heating at 80°C for 1 min. After the reaction, the products were partitioned between chloroform and aqueous methanol by the procedure of Bligh and Dyer,[25] and 2 ml of the chloroform phase was concentrated and spotted on Silica Gel G plates (Merck). After TLC, each fraction of FFA, PC and LPC was scraped off, and its radioactivity was counted in a Packard Tri-Carb 2425 liquid scintillation spectrometer with 10 ml of scintillation mixture containing 0.1 g of POPOP and 4 g of PPO per liter of toluene.

Disc electrophoresis. Disc electrophoresis was conducted in 7.5% gel for 90 min with a current of 2 mA/tube at 4°C in accordance with the method of Ornstein *et al.* (pH 9.4).[49] Protein band was stained with Amide Black 10 B.

Thin layer-chromatography, isoelectric focusing and determination of protein concentration and molecular weight. All of these procedures are the same as that described in Chapter II.

Results

Purification of LPLase

Step 1. Desalting. The crude enzyme was desalted by gel filtration with Bio-gel P-10.

Step 2. First DEAE-Sephadex column chromatography. The desalted solution was applied to a DEAE-Sephadex A-25 column (Fig. 1). LPLase activity was separated into three fractions. The most

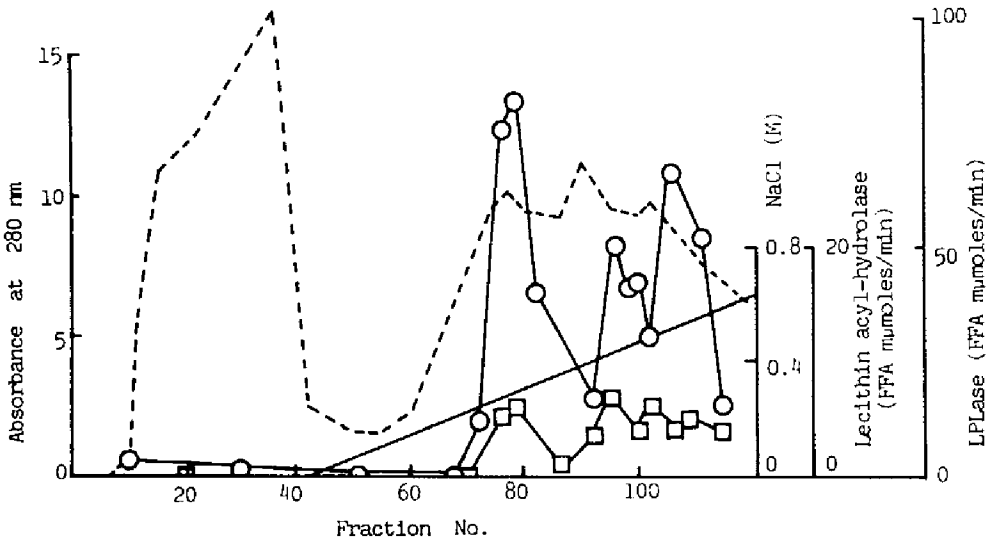


Fig. 1. DEAE-Sephadex Column Chromatography of LPLase from *C. centrifugum.*

Desalted enzyme solution (320 ml, 6.72 g protein) was applied to a DEAE-Sephadex A-25 column (2.6 x 36 cm), which was equilibrated with 0.05 M Tris-HCl buffer, pH 7.3. Elution was carried out at a rate of 30 ml/hr with a linear gradient of NaCl concentration from zero to 0.8 M in the same buffer, and 10 ml fractions were collected.

- - - - - -, absorbance at 280 nm; O—O , LPLase activity; □—□ , lecithin acyl-hydrolase activity; ———— , NaCl concentration.

active fractions (tube Nos. 73-86) were combined, and concentrated with collodion-bag. The concentrated solution was dialyzed against 0.05 M Tris-HCl buffer, pH 7.3.

Step 3. Second DEAE-Sephadex column chromatography. The dialyzed solution was rechromatographed on a DEAE-Sephadex A-25 column. The active fractions were combined, and concentrated with collodion-bag. The concentrated solution was dialyzed against 10 mM phosphate buffer, pH 7.1.

Step 4. Hydroxylapatite column chromatography. The dialyzed solution was applied to a hydroxylapatite column (Fig. 2).

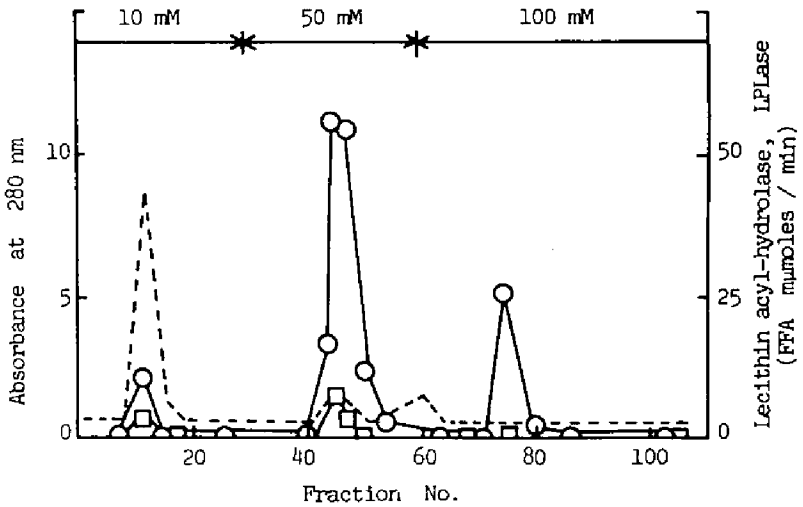


Fig. 2. Hydroxylapatite Column Chromatography of LPLase.

The dialyzed solution (8 ml, 132 mg protein) from the second DEAE-Sephadex (No. 51-57) was applied to a hydroxylapatite column (2.6 x 10 cm), which was consisted of hydroxylapatite and cellulose (1 : 1, v/v), and was equilibrated with 10 mM phosphate buffer, pH 7.1. Elution was carried out at a rate of 35 ml/hr with a stepwise increase in buffer concentration (10, 50 and 100 mM) and 5 ml fractions were collected.

- - - - -, absorbance at 280 nm; O—O , LPLase activity; □—□ , lecithin acyl-hydrolase activity.

The activity was eluted at 50 mM and 100 mM buffer. The active fractions were concentrated and named 50 mM Frn. and 100 mM Frn., respectively. After concentration with collodion-bag, these fractions were preserved at -20°C.

The results of purification by above procedures are summarized in Table I. The 50 mM Frn. and 100 mM Frn. showed 37- and 92-fold

Table I. Summary of the Purification of LPLase Produced by *C. centrifugum*

Procedure	Protein (mg)	Activity (units)		Specific activity (units/mg)	
		LPLase	Lecithin acyl-hydrolase	LPLase	Lecithin acyl-hydrolase
Desalted solution of lyophilized protein of $(NH_4)_2SO_4$ ppt. (100% satn.)	6720	4964.4	5325.6	0.74	0.79
1st DEAE-Sephadex	875	1632.8	430.5	1.86	0.49
2nd DEAE-Sephadex	131.6	781.3	109.6	5.55	0.83
Hydroxylapatite					
50 mM Frn.	11.6	313.9	39.4	27.18	3.41
100 mM Frn.	1.3	91.1	0.0	68.00	0.00

increase in specific activity, respectively. The 100 mM Frn. was used for the investigation of various properties described below except for the estimation of molecular weight. The hydrolysis reaction showed a linear rate up to at least 10 min incubation by the standard assay method.

Homogeneity of purified enzyme

Homogeneities of both fractions were examined by disc electrophoresis (pH 9.4). The 50 mM Frn. had two LPLase components, and 100 mM Frn. had one (Fig. 3-(a) and (b)). The LPLase component of

50 mM Frn. (active fraction B in Fig. 3-(a)) was found a little contaminated with lecithin acyl-hydrolase activity.

Estimation of molecular weight

The molecular weight of LPLase (50 mM Frn.: active fraction A in Fig. 3-(a)), determined by gel filtration with Sephadex G-200 (Fig. 4), was estimated to be about 130,000.

Isoelectric point

50 mM Frn. (active fraction A in Fig. 3-(a)) and 100 mM Frn. had the same isoelectric point at about pH 3.9.

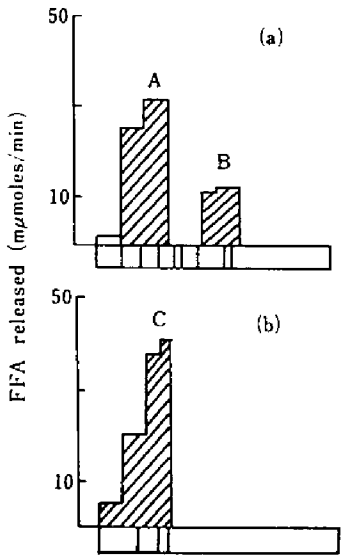


Fig. 3. Disc Electrophoresis of LPLase.

About 30~50 μg of the purified enzyme was subjected to electrophoresis at pH 9.4. See the text for experimental details. (a) and (b) are the patterns of disc electrophoresis of 50 mM Frn. and 100 mM Frn., respectively.

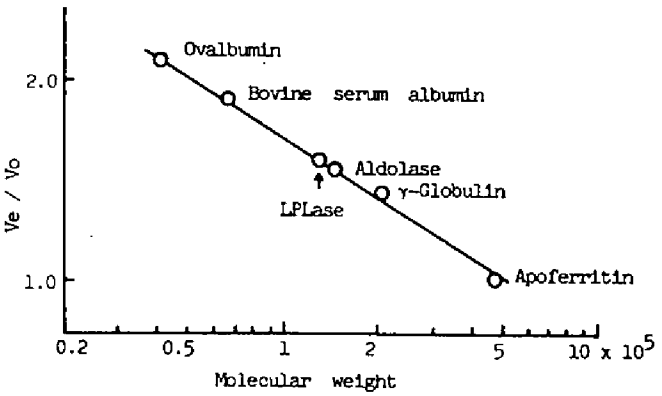


Fig. 4. Estimation of Molecular Weight of LPLase by Gel Filtration.

Chromatography was carried out on a Sephadex G-200 column (1.5 x 95 cm) with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M KCl after the column had been equilibrated with the same buffer. Ve, the elution volume; Vo, the void volume.

Optimal pH

As shown in Fig. 5, the pH optimum of LPLase activity was found to be 3.5-5.0.

pH stability

The stability of LPLase in solution of various pH values is shown in Fig. 6. The enzyme was most stable in the pH range of 7.0 to 8.0, and more stable on the acidic than on the alkaline side.

Thermal stability

The enzyme was stable up to 35°C at pH 7.0, but it rapidly lost its activity at 45°C. Up to 40°C, the enzyme was more stable at pH 7.0 than at pH 4.0, but from 40 to 60°C, it was more stable at pH 4.0 (Fig. 7).

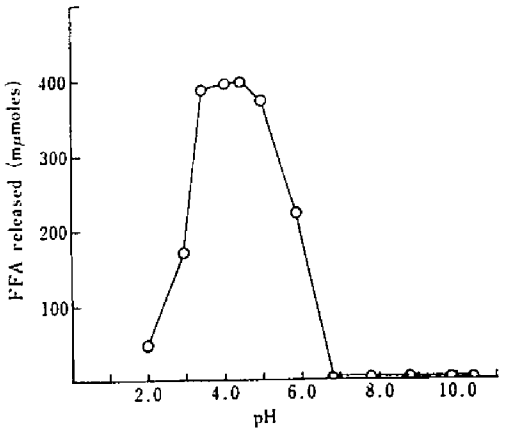


Fig. 5. Effect of pH on LPLase Activity.

The enzyme activity was measured by the standard assay method with enzyme preparation I. Buffers used were: 0.1 M glycine-HCl buffer containing 0.1 M NaCl (pH 2.0~2.9), 0.1 M acetate buffer (pH 3.4~5.8), 0.03 M phosphate buffer (pH 6.8~7.9) and 0.1 M glycine-NaOH buffer containing 0.1 M NaCl (pH 8.8~10.6).

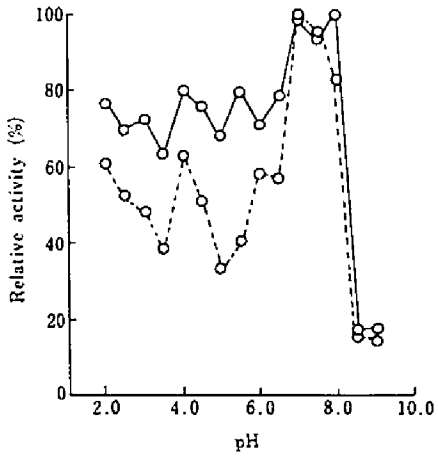


Fig. 6. Effect of pH on the Stability of LPLase.

The enzyme solution was incubated at each pH value from 2.0 to 9.0 for 47 and 100 hr at 4°C, and the remaining activities were assayed. Buffers used were: 0.1 M sodium acetate-HCl buffer (pH 2.0~3.0), 0.1 M acetate buffer (pH 3.5~5.0) 0.05 M phosphate buffer (pH 5.5~8.0) and 0.05 M Tris-HCl buffer (pH 8.5~9.0).

○—○, incubation for 47 hr; ○---○, incubation for 100 hr.

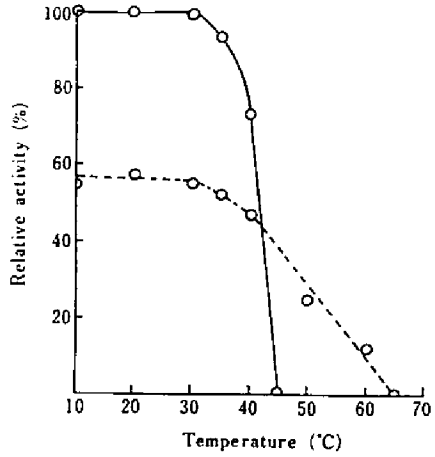


Fig. 7. Thermal Stability of LPLase.

The enzyme solution was incubated at various temperatures for 15 min at pH 4.0 and 7.0, and the remaining activity was assayed.
○—○, pH 7.0; ○--○, pH 4.0.

Effect of metal ions

The effect of various metal ions on LPLase activity is shown in Table II. None of the metal ions stimulated LPLase activity. Fe^{3+}, Hg^{2+} and Al^{3+} inhibited 100%, 46% and 32% of LPLase activity, respectively.

Table II. Effect of Metal Ions on LPLase Activity

The enzyme activity was determined by the standard assay method with enzyme preparation I, except that 10 mM of one or another of the indicated reagents was added before the reaction. The activity is expressed in percentage of the activity level in the absence of metal salts. Ag^+ was used as the nitrate form and the other metal ions were used as the chloride form.

Metal ions (10 mM)	Activity (%)
None	100
Mg^{2+}	93
Ca^{2+}	93
Ba^{2+}	98
Al^{3+}	68
Mn^{2+}	102
Fe^{2+} [a]	101
Fe^{3+}	3
Co^{2+}	98
Ni^{2+}	94
Cu^{2+}	94
Zn^{2+}	92
Ag^+	101
Hg^{2+}	54

[a] Fe^{2+} was prepared from $FeCl_2 \cdot nH_2O$ ($n=6$).

Effect of organic solvents

Enzyme activity was measured by incubation with various organic solvents (Table III). Ethanol, methanol, 1-propanol, 2-propanol and glycerol at a final concentration of 10% stimulated LPLase activity, but ethanol at 30% inhibited the activity. When the enzyme solution was incubated with 5 - 20% ethyl ether, enzyme activity was stimulated by 20-30%.

Table III. Effect of Organic Solvents on LPLase Activity

The enzyme activities were determined by the standard assay method with enzyme preparation I, except that one or another of the listed organic solvents at the indicated concentration was added before the reaction. When the volume of organic solvent had to be increased, the equivalent volume of acetate buffer was decreased.

Organic solvents	Conc. (%)	Activity (%)
None	—	100
Ethanol	10	161
	20	153
	30	41
Methanol	10	200
1-Propanol	10	142
2-Propanol	10	196
Glycerol	10	124

Effect of reagents

The effect of various reagents on LPLase activity is shown in Table IV. Despite the inhibition of enzymatic activity by Hg^{2+}, LPLase was not inhibited by specific SH reagents. Among imidazole group reagents, diethylpyrocarbonate did not inhibit LPLase activity, but *p*-diazobenzenesulfonic acid did inhibit 89% of it at 10 mM. *N*-Bromosuccinimide exhibited an inhibitory effect of 100% at 1 mM. Among serine-OH group reagents, diisopropylfluorophosphate inhibited LPLase by 19% at 1 mM concentration.

Table IV. Effect of Various Reagents on LPLase Activity

The enzyme was incubated with each reagent in 0.05 M phosphate buffer, pH 7.0, at 30°C for 30 min, and the residual activity was assayed. When diethylpyrocarbonate was used, the enzyme solution was incubated in 0.05 M phosphate buffer, pH 7.0, containing ethanol at 0°C for 30 min. When diisopropylfluorophosphate and phenylmethylfluoride were used, the enzyme solution was incubated in 0.1 M acetate buffer, pH 4.0, containing isopropanol at 0°C for 60 min.

Addition	Concentration (mM)	Inhibition (%)
N-Ethylmaleimide	10	3
Dithionitrobenzoic acid	1	4
p-Chloromercuribenzoate	1	0
Iodoacetamide	10	5
Monoiodoacetic acid	10	0
N-Acetylimidazole	10	0
1,2-Cyclohexanedione	10	0
Diethylpyrocarbonate	10	0
p-Diazobenzenesulfonic acid	10	89
N-Bromosuccinimide	1	100
Diisopropylfluorophosphate	1	19
Phenylmethylsulfonylfluoride	1	7

Substrate specificity

Apparent Michaelis constants (*Km*) of LPLase activity for [U-^{14}C]-1-acyl-LPC, [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC were calculated from Lineweaver-Burk plots to be 0.35, 0.16 and 0.09 mM, and *Vmax* were 79.1, 58.7 and 52.1 μmoles/min per mg of protein, respectively, as shown in Fig. 8. The Lineweaver-Burk

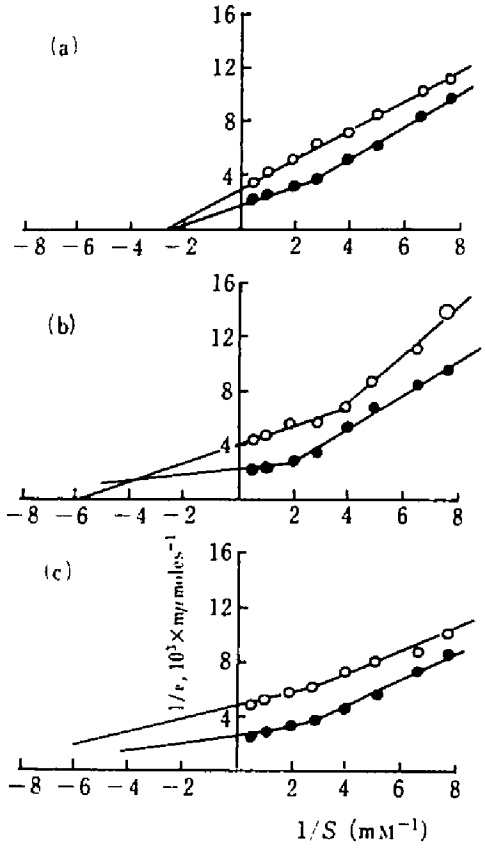


Fig. 8. Effect of LPC Concentration on LPLase Activity.

The enzyme activities were determined by standard methods. (a) [U-^{14}C]-1-acyl-LPC, (b) [1-^{14}C]-palmitoyl-LPC, (c) [1-^{14}C]-oleoyl-LPC. O—O, without ethanol; ●—●, with 10% ethanol.

plots for [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC were broken at 0.25 mM and 0.4 mM, respectively. Under the influence of 10% ethanol, their Michaelis constants were 0.36, 0.07 and 0.07 mM, and *Vmax* were 131.6, 106.4 and 96.2 μmoles/min per mg of protein,

respectively. In this case , the Lineweaver-Burk plot for [U-^{14}C]-1-acyl-LPC was also broken at 0.34 mM, and the break points for [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC rose.

Mono-, di- and tripalmitin were used as substrates for lipase activity. These substrates were used as a dispersed solution by a ultrasonication or with 0.1% Triton X-100 or 10% ethyl ether. The enzyme solution was incubated with 2 mM substrates in acetate buffer, pH 4.0, at 30°C for 30 min, but these substrates were not hydrolyzed.

PC was used as substrate for lecithin acyl-hydrolase activity. The enzyme was incubated with an ultrasonic dispersion of PC (1 mM) in the acetate buffer, pH 4.0, at 30°C for 30 min, but PC was not hydrolyzed.

Effect of bovine serum albumin

The effect of bovine serum albumin on LPLase activity was investigated, with [U-^{14}C]-1-acyl-LPC, [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC used as substrates. LPLase activity was not affected by bovine serum albumin below 500 μg/ml, regardless of the kind of substrate.

Effect of phosphatidylcholine

The enzyme solution was incubated with 1 mM labeled LPC and various concentrations of PC. As shown in Fig. 9, regardless of the kind of substrate, LPLase activity was little affected by PC.

Effect of detergents

The effect of detergents was investigated with various substrates as shown in Fig. 10. When [U-^{14}C]-1-acyl-LPC was used as substrate, LPLase activity was stimulated by Triton X-100 at a concentration of 0.1% (w/v) and by Tween 80 at 0.1 and 0.4%. However, when [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC were used as substrates, LPLase activity was inhibited by both detergents at all concentrations tested.

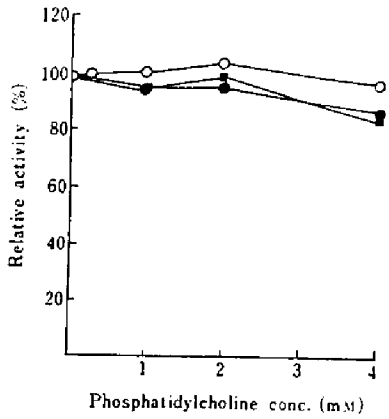


Fig. 9. Effect of Phosphatidylcholine on LPLase Activity.

The enzyme activities were determined by standard methods except that varying amounts of ultrasonic dispersion of PC were added before the reaction. Substrates: O—O, [U-^{14}C]-1-acyl-LPC; ●—●, [1-^{14}C]-palmitoyl-LPC; ■—■, [1-^{14}C]-oleoyl-LPC.

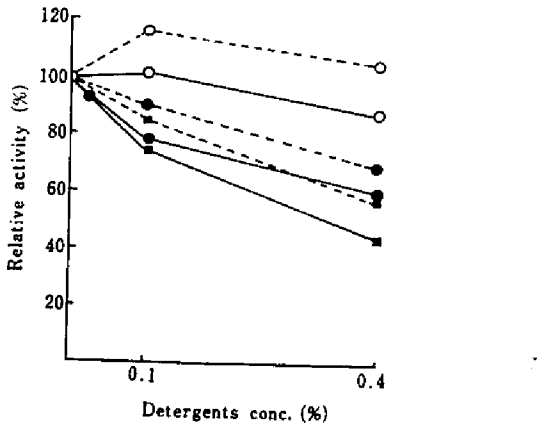


Fig. 10. Effect of Detergents on LPLase Activity.

The enzyme activities were determined by standard methods except that varying concentrations of detergents were added before the reaction. Substrates: O—O, [U-^{14}C]-1-acyl-LPC; ●—●, [1-^{14}C]-palmitoyl-LPC; ■—■, [1-^{14}C]-oleoyl-LPC. Detergents: - - - - -, Tween 80; ———, Triton X-100.

Discussion

In the hydroxylapatite column chromatography, the active fraction was divided into 50 mM Frn. and 100 mM Frn. However, the properties of these fractions with respect to the pattern of disc electrophoresis, optimal pH, isoelectric point, effects of stimulators and inhibitors and *Km* for LPC were similar, and it was not found any difference. The molecular weight of LPLase was determined with 50 mM Frn.

LPLase produced by *C. centrifugum* is similar to that of the mold, *Penicillium notatum*,[47] in acidic pH optimum (3.5 - 5.0), isoelectric point (pH 3.9), molecular weight (130,000) and thermal instability.

Inhibition by histidine reagents was observed with LPLase from cod muscle,[50] and diisopropylfluorophosphate inhibition was observed with bovine pancreas,[51] but no inhibition by *N*-bromosuccinimide has been reported.

This enzyme differed from those of the bovine pancreas[51] and *E. coli*[52] enzymes which show the lipase activity. Nor did it exhibit PLase B activity which was observed with *P. notatum*.[47]

LPLase activity of *C. centrifugum* was not affected by bovine serum albumin. This result is similar to that observed with the LPLase of *Culex pipiens fatigans*.[53]

The inhibition of LPLase by PC, which was observed with bovine pancreas[51] and mosquito,[53] was not observed with *C. centrifugum*.

The sensitivity of LPLase from *C. centrifugum* to stimulation by various organic solvents and detergents characteristically distinguishes this enzyme from other general LPLases.

The appearance of a break point and the difference of its values with each three substrates which is different in its fatty acid composition, in Lineweaver-Burk plots mean that the micellar form gives a better substrate for LPLase than the monomolecular form, and that each LPC has its own micellar structure determined by its fatty acid composition. Under the influence of 10% ethanol, though *Km* did not change, *Vmax* was greater than in the absence of ethanol. This result suggests that ethanol mainly affects the enzyme rather than the substrate in the hydrolysis of LPC. [U-^{14}C]-1-Acyl-LPC was hydrolysed more rapidly than [1-^{14}C]-palmitoyl-LPC and [1-^{14}C]-oleoyl-LPC. The fatty acid composition of a carrier LPC of [U-^{14}C]-1-acyl-LPC is shown in Table V. This indicates that the mixed composition of fatty acids of a carrier LPC gives a good micellar structure for this enzyme to [U-^{14}C]- 1-acyl-LPC.

TABLE V. MOLECULAR SPECIES OF LPC

Carbon number	Fatty acid C-1	C-2	wt (%)
16	16:0 (Palmitic acid)	—	79.3
18	18:0 (Stearic acid)	—	16.6
	18:1 (Oleic acid)	—	4.1

In the experiment concerning the effect of detergents on LPLase activity, the change of substrate from [U-^{14}C]-1-acyl-LPC to [1-^{14}C]-palmitoyl-LPC or [1-^{14}C]-oleoyl-LPC was observed to reverse

the effect of detergents. This result indicates that the interaction of LPC and detergents is affected by the fatty acid composition of LPC, and [U-^{14}C]-1-acyl-LPC that has a mixed composition of fatty acids can be more favorable to LPLase.

Section IV-2. Purification and Properties of Lysophospholipase Produced by *C. centrifugum*: General Properties and Determination of Reaction Type

In this Section, the term LPLase 1,2 is used for the enzyme that hydrolyzes the acyl ester bonds at both position 1 and 2 of lysophospholipid molecule.

Materials and Methods

Enzyme. The hydroxylapatite-purified enzyme preparation, which was described in Chapter III, was used.

Substrate. 1- or 2-Acyl-LPC were prepared as described in Chapter II, and 1-acyl-LPC was generally used as a substrate for the assay of LPLase activity.

Enzyme assay. Assay method and the definition of unit of LPLase activity were the same as that described in Chapter II.

Thin-layer chromatography, isoelectric focusing and determination of protein concentration and molecular weight. All of these procedures were the same as that described in Chapter II.

Results

Purification of LPLase

The purification procedures and their results are described in Chapter III.

Homogeneity of LPLase activity of the enzyme preparation purified by hydroxylapatite

The hydroxylapatite-purified enzyme preparation was applied to a Sephadex G-200 column (Fig. 2 in Chapter III). This enzyme preparation contained one component of lecithin acyl-hydrolase activity, tube Nos. 42-50, and two components of LPLase activity, as a peak, tube Nos. 34-40, and a shoulder, tube Nos. 42-50. The homogeneity of the main LPLase active fraction was estimated as about 90% from the area of the activity peaks. The isoelectric focusing showed that the main LPLase active fraction was composed of only one component. The isoelectric point of LPLase was found to be 3.92.

Estimation of the molecular weight

The molecular weight of LPLase was estimated to be about 100,000 by the gel filtration as shown in Fig. 1.

Optimal pH

As shown in Fig. 2, the optimal pH of LPLase activity was found to be 4.0.

pH stability

As shown in Fig. 3, LPLase was most stable at pH 5.5-8.0, and more stable on the acidic side than on the alkaline side.

Thermal stability

LPLase activity was rapidly lost at 45°C, pH 7.0 (Table II in Chapter III).

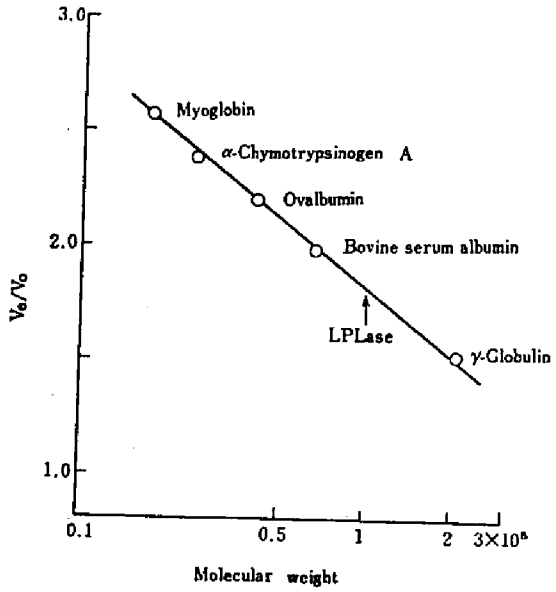


Fig. 1. Estimation of Molecular Weights of LPLase by Gel Filtration.

Chromatography was carried out on a Sephadex G-200 column (1.5 x 92 cm) with 0.05 M Tris-HCl buffer, pH 7.2, containing 0.1 M KCl, after the column had been equilibrated with the same buffer, and 2 ml fractions were collected.
Ve, the elution volume; Vo, the void volume.

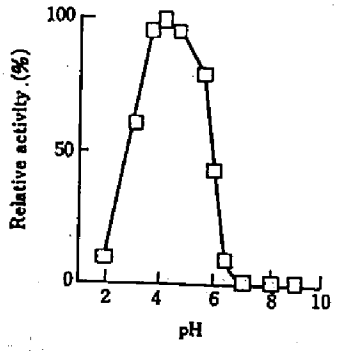


Fig. 2. Optimal pH of LPLase Activity.

The enzyme activity was measured by the standard assay method in the following buffers: 0.1 M sodium acetate-HCl buffer (pH 2.0 - 3.0), 0.1 M acetate buffer (pH 3.5 - 5.0), 0.2 M Tris-malate-NaOH buffer (pH 5.5 - 8.0) and 0.05 M Tris-HCl buffer (pH 8.5 - 9.0).

Determination of the type of reaction of LPLase

The enzyme preparation used was the main LPLase fraction, tube Nos. 34-40 in the experiment of gel filtration (Fig. 2 in Chapter III). Both 1- and 2-acyl-LPC were fully hydrolyzed, and 2-acyl-LPC was hydrolyzed slightly better (Table I). It was confirmed that isomerization of 2-acyl-LPC to 1-acyl-LPC did not take place under this assay condition at pH 4.0 (unpublished data). From these results, it was found that this LPLase was classified into the type of LPLase 1,2.

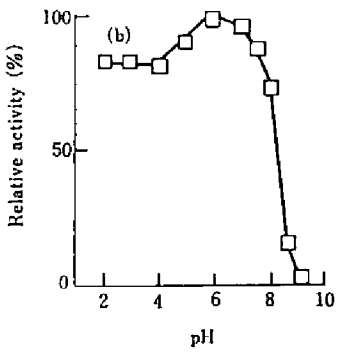


Fig. 3. pH Stability of LPLase Activity.

The enzyme solution was incubated at each pH value for 45 hr at 4°C, and the remaining activities were assayed. Buffers used were 0.1 M sodium acetate-HCl buffer (pH 2.0 - 3.0), 0.1 M acetate buffer (pH 3.5 - 5.0), 0.2 M Tris-malate-NaOH buffer (pH 5.5 - 8.0) and 0.05 M Tris-HCl buffer (pH 8.5 - 9.0).

Table I. Determination of the Type of Reaction of LPLase

The enzyme activity was measured by the standard assay method using 1-acyl-LPC and 2-acyl-LPC as substrate. The enzyme used was the one which recovered from tube Nos. 34 to 40 in the experiment of gel filtration (Fig. 2. in Section IV-1 of Chapter III).

Substrate	Free fatty acid released (nmole, (%))
1-Acyl-LPC	538 (100)
2-Acyl-LPC	640 (118)

Effect of reagents

The effects of various reagents on LPLase activity is shown in Table II. EDTA and SH reagents did not affect LPLase activity. But, *p*-diazobenzenesulfonic acid and *N*-bromosuccinimide inhibited the enzyme activity.

Table II. Effects of Various Reagents on LPLase Activities.
The enzyme was incubated with each reagent in 0.05 M phosphate buffer, pH 7.2, at 30 C for 30 min, and the residual activity was measured by the standard assay method.

Reagent	Conc. (mM)	Activity (%)
None	-	100
EDTA	10	104
N-Ethylmaleimide	10	105
Monoiodoacetic acid	10	103
Iodoacetamide	10	101
p-Diazobenzenesulfonic acid	1	63
	10	10
N-Bromosuccinimide	1	0

Effects of organic solvents and detergents

As shown in Table III, LPLase activity was stimulated up to about 1.9-fold. But it was completely inhibited by 0.1% sodium dodecyl sulfate,

Table III. Effects of Organic Solvents and Detergents on LPLase Activity

The enzyme activity was measured by the standard assay method except that one of the listed organic solvents and detergents at the indicated concentration was added before the reaction.

Reagent	Conc. (%)	Activity (%)
None	-	100
Ether	10	134
Methanol	10	170
Ethanol	10	188
1-Propanol	10	179
2-Propanol	10	188
Acetone	10	187
Sodium dodecyl sulfate	0.1	0
Cetyltrimethylammonium bromide	0.1	31
Triton X-100	0.1	90
	0.4	65
Tween 80	0.1	95
	0.4	78
Tween 20	0.1	104
	0.4	87

and generally received inhibitory effects by nonionic and cationic detergents.

Effects of metal ions

LPLase activity was inhibited about 40% by Fe^{2+} and Fe^{3+} (Table IV).

Table IV. Effects of Metal Ions on LPLase Activity

The enzyme activity was measured by the standard assay method except that 10 mM of one of the indicated reagents was added before the reaction. The activity is expressed in percentage of the activity level in the absence of metal salts. Fe^{2+} was used as the sulfate form, and the other metal ions were used as the chloride form.

Metal ion (10 mM)	Activity (%)
None	100
Na^{+}	97
Ba^{2+}	92
Ca^{2+}	99
Mg^{2+}	95
Mn^{2+}	95
Fe^{2+}	59
Cu^{2+}	80
Zn^{2+}	97
Cd^{2+}	98
Hg^{2+}	77
Fe^{3+}	58
Al^{3+}	76

Km value

The apparent Michaelis constant of LPLase activity was calculated from the Lineweaver-Burk plot to be 0.24 mM (Fig. 4). The Lineweaver-Burk plot broke upward at 0.31 mM concentration of LPC, which corresponded to the critical micelle concentration of LPC.

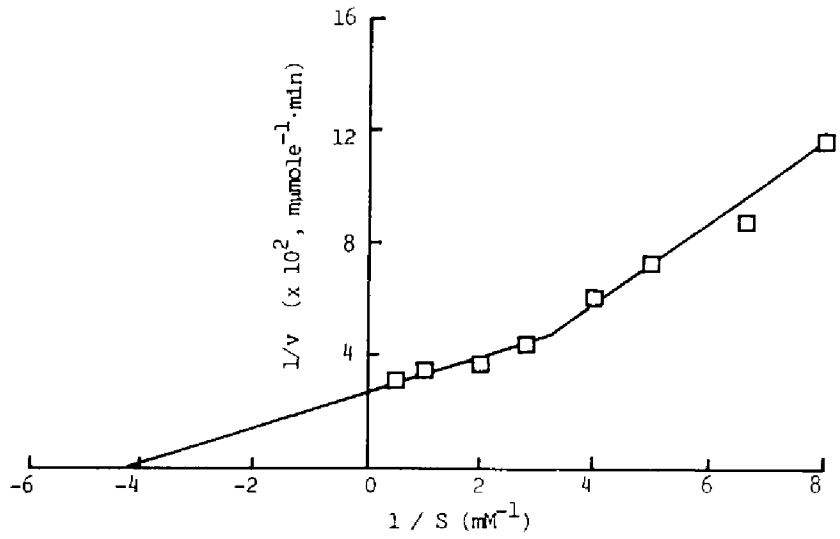


Fig. 4. Effect of LPC Concentration on LPLase Activity.

The enzyme activity was measured by the standard assay method.

Discussion

Since all the enzyme activities throughout PLase A_1, LPLase 1, PLase B and LPLase 1,2 were inhibited by *p*-diazobenzenesulfonic acid and *N*-bromosuccinimide, it was suggested that in spite of the different enzyme activities, these enzyme activities were realized by similar active sites.

It was found that LPLase 1,2 was similar to LPLase, which was described in Section IV-1 of Chapter IV, in its properties such as the elution point in DEAE-Sephadex column chromatography, specific activity (50 mM Frn. in Section IV-1), isoelectric point, optimum pH, pH stability, heat stability, effect of reagents, stimulation

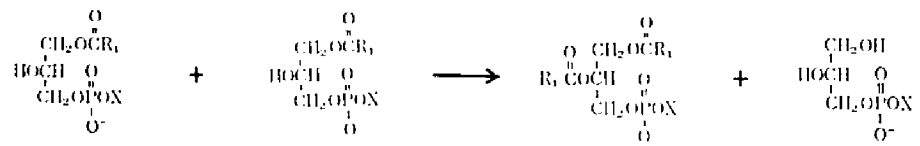
by organic solvents, substrate specificity and *Km*, although there were some difference in the molecular weight, degree of inhibition by Fe^{2+} and Fe^{3+}, and the degree of the effect of detergents. So it seems that these enzymes are the same.

When the first DEAE-Sephadex column chromatograms of Section IV-1 in Chapter IV and of Chapter II or III are compared, it is found that LPLase active fraction, which is possessed by PLase A_1 and should be eluted with the NaCl concentration of 0.45 M, almost vanishes in the former. This disappearance seems to be resulted from its pH instability (Fig. 3 in Chapter II), and may be denatured during the procedure of ultrafiltration for about 8 hr.

CHAPTER V

OCCURRENCE OF LYSOLECITHIN-LYSOLECITHIN ACYLTRANSFERASE ACTIVITY IN LYSOPHOSPHOLIPASE PRODUCED BY *CORTICIUM CENTRIFUGUM*

LPLase (EC 3.1.1.5) produced by *Corticium centrifugum* was purified and investigated of its properties as described in Section IV-1 of Chapter IV. In the course of this study, it was observed that PC was synthesized in the reaction products from LPC. The enzyme reaction was carried out by use of purified enzyme preparation under the standard assay conditions described below. The existence of PC was proved by the positive reaction against phosphate[43] and choline[54] detection reagents and its Rf value on TLC (chloroform-methanol-water; 80:35:5, v/v/v). The reaction mixture contained neither CoA nor ATP, so that it was presumed that the reaction would be performed as follows:



LPC + LPC → PC + GPC

R_1CO-, fatty acyl residues; X, choline moiety.

This enzyme activity has been named LPC-LPC acyltransferase activity,[55] and reported in rat liver[55-57] and lung supernatant,[58] yeast supernatant,[59] homogenates of rabbit erythrocytes[60] and polymorphonuclear leukocytes.[61] However, the example purified has not been reported as yet except for rat-lung soluble fraction.[62] Abe *et al.*[62] partially purified LPC-LPC acyltransferase activity

from this soluble fraction and suggested for the first time that the same enzyme was responsible for both LPLase and LPC-LPC acyltransferase activities. But, further purification of the enzyme has been hampered by the instability of partially purified enzyme preparation and its properties have not been adequately investigated.

In this Chapter, the phenomenon that LPC-LPC acyltransferase activity comes into the appearance in LPLase by a certain modification and the properties of LPC-LPC acyltransferase activity are described.

Materials and Methods

Enzyme. *Corticium centrifugum* IAM 9028 was cultured in a medium, consisting of heat-treated brewer's yeast. The culture broth was filtered and concentrated by ultrafiltration. All proteins were collected by saturation with ammonium sulfate. From this protein fraction, the enzyme was purified twice by column chromatography with DEAE-Sephadex, and with hydroxylapatite. The purified enzyme fraction, eluted from hydroxylapatite column with 100 mM phosphate buffer at pH 7.4, showed 92-fold purification in LPLase activity. The fraction contained no activity of PLase A or B. Disc electrophoresis (pH 9.4) indicated that this enzyme preparation had a few protein bands stained with Amide Black 10 B, but had only one LPLase component as phospholipid acyl-hydrolase.

Substrate. LPC and [U-^{14}C]-1-acyl-LPC were prepared as described in Section IV-1 of Chapter IV.

Enzyme assay. When cold LPC was used as substrate, LPC-LPC acyltransferase activity was measured by the area of PC spot on TLC. When [U-^{14}C]-1-acyl-LPC was used as substrate, LPLase and LPC-LPC acyltransferase activities were measured by the amounts of FFA released and of PC formed as follows: the reaction mixture contained 1 μmole of [U-^{14}C]-1-acyl-LPC, 5 x 10^4 dpm/μmole, 0.5 ml of 0.1 M acetate buffer, pH 3.6, 0.35 ml of water and 0.15 ml of enzyme solution in a total volume of 1 ml, and was incubated at 45°C for 8 min with shaking at a rate of 120 times per min. The reaction was stopped by the addition of 2.5 ml of methanol and by heating at 80°C for 1 min, and then the products were extracted by the procedure of Bligh and Dyer,[25] and 2 ml of the chloroform phase was concentrated and spotted on Silica Gel G plates (Merck). After TLC (chloroform-methanol-water; 80:35:5, v/v/v), each fraction of FFA, PC and LPC was scraped off and its radioactivity was counted in a Packard Tri-Carb 2425 liquid scintillation spectrometer with 10 ml of scintillation mixture containing 0.1 g POPOP and 4 g PPO per liter of toluene. FFA, PC and LPC had the same counting efficiency, 32%.

Disc electrophoresis. Disc electrophoresis was carried out according to the method of Ornstein *et al.* (pH 9.4)[49] and Williams *et al.* (pH 8.0).[33]

Isoelectric focusing. Isoelectric focusing was carried out as described in Chapter II.

Results and Discussion

When the LPC-LPC acyltransferase activity was first observed on TLC, it was very weak in comparison with LPLase activity, less than one hundredth (Preparation I). But in one of purified enzyme preparations, it was observed that the former activity increased with the number of days during the preservation in 50 mM phosphate buffer, pH 7.0, at 0°C after the storage at -20°C (Fig. 1). At 30 days after thawing, the ratio of the reaction velocity of LPLase activity to LPC-LPC acyltransferase activity became 6 : 1. We have examined the properties of LPC-LPC acyltransferase activity by using this enzyme preparation (Preparation II). It was observed on disc electrophoresis that the mobility of LPLase (Preparation II) was different from that of LPLase (Preparation I). The position of LPC-LPC acyltransferase

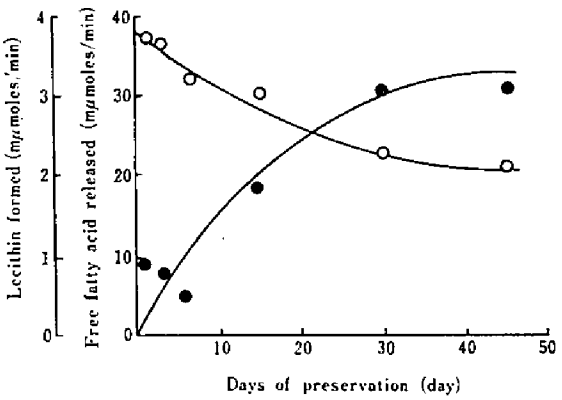


FIG. 1. Effect of Days of Preservation on Lysophospholipase and LPC-LPC Acyltransferase Activities.
The enzyme was preserved in 50 mM phosphate buffer, pH 7.0, at 0°C. The enzyme activities were determined with days by standard assay method.
○—○, lysophospholipase activity; ●—●, LPC-LPC acyltransferase activity.

activity on disc gel, however, coincided always with that of LPLase activity. Both activities could not be separated by isoelectric focusing. LPC-LPC acyltransferase activity had the optimum pH at 4.0. It was most stable at neutral pH, unstable against heating, and was inhibited by Fe^{3+}, Hg^{2+} and Al^{3+}. Such properties of LPC-LPC acyltransferase activity were the same as those of LPLase activity. The apparent Michaelis constants for LPLase and LPC-LPC acyltransferase activities were 0.78 and 0.58 mM, respectively. The maximum reaction velocity of LPLase activity was about 6.6-fold faster than that of LPC-LPC acyltransferase activity. LPLase activity was not affected by PC and bovine serum albumin, and was stimulated by various organic solvents such as methanol, propanol *etc.*, and detergents such as Triton X-100 and Tween 80, as described in Section IV-1 of Chapter IV. On the other hand, LPC-LPC acyltransferase activity was inhibited by all these substances.

The effectiveness of several treatments on the increase of LPC-LPC acyltransferase activity was investigated. Freezing at -20°C and treatments with urea or with reducing and oxidizing reagents were not effective.

The above results suggest that both LPLase and LPC-LPC acyltransferase activities are carried by the same enzyme protein, and the appearance of LPC-LPC acyltransferase activity may be caused by a certain modification. This enzyme preparation is not pure, so that there is a possibility that LPLase receives a limitted

proteolysis. Another possibility is that some substance, such as phosphate, may be bound to LPLase or liberated from it. But, these possibilities have not been proved experimentally. The physiological significance of LPC-LPC acyltransferase activity in *Corticium centrifugum* is not clear.

CHAPTER VI

DISCUSSION AND SUMMARY

Chapter II;

PLase A_1, purified from the culture filtrate of *C. centrifugum*, was found to possess LPLase 1 activity. The isoelectric point was pH 3.3, and the molecular weight was about 26,800. Both enzyme activities had their pH optimum between 4.0 and 4.5 and their pH stability between 6.0 and 8.0, and were heat-unstable. Both were inhibited by *p*-diazobenzenesulfonic acid and *N*-bromosuccinimide. Although ether, 1-propanol and Triton X-100 stimulated PLase A_1 activity, these substances showed an inhibitory effect against LPLase 1 activity. PLase A_1 activity was almost completely inhibited by Fe^{2+}, Fe^{3+} and Al^{3+}, but the inhibition was lessened by the presence of Triton X-100. LPLase 1 activity was inhibited by Hg^{2+}, Fe^{3+} and Al^{3+}. The mode of inhibition by Fe^{3+} against LPLase 1 activity was apparently an uncompetitive type. PLase A_1 hydrolyzed various phospholipids, but not triolein.

Chapter III;

Lecithin acyl-hydrolase was partially purified from the culture filtrate of *C. centrifugum*. The heat treatment examination and analysis of the reaction products demonstrated that this lecithin acyl-hydrolase was PLase B. The isoelectric point of PLase B was pH 4.04 and the molecular weight was about 48,000. PLase B had an

optimal pH 5.5 for the reaction, and received an apparent uncompetitive inhibition by Fe^{2+}, Fe^{3+} and Al^{3+}. *p*-Diazobenzenesulfonic acid, *N*-bromosuccinimide, organic solvents and detergents also inhibited PLase B activity. PLase B showed a wide substrate specificity about the various phospholipids contained in plant tissues.

Chapter IV

Section IV-1;

LPLase (EC 3.1.1.5) from the culture broth of *C. centrifugum* was purified 92-fold in specific activity by DEAE-Sephadex and hydroxylapatite column chromatography. The isoelectric point was at about pH 3.9, and the molecular weight was about 130,000. The optimal pH was about 3.5-5.0. The stable pH range was from 7.0 to 8.0. LPLase activity was inhibited by Fe^{3+}, Hg^{2+} and Al^{3+}, but stimulated by various organic solvents. *p*-Diazobenzenesulfonic acid, *N*-bromosuccinimide and diisopropylfluorophosphate also inhibited the activity. This enzyme did not hydrolyze mono-, di- or tripalmitin or PC. Apparent Michaelis constants of LPLase activity for 1-acyl-LPC, 1-palmitoyl-LPC and 1-oleoyl-LPC were 0.35, 0.16 and 0.09 mM, respectively. The effect of detergents on the enzyme activity was observed to differ with the fatty acid composition of substrate.

Section IV-2;

In this examination, differring from Section IV-1, enzyme proteins which were collected from the culture filtrate by the

fractionation with ammonium sulfate without ultrafiltration were used as a crude enzyme preparation. Although between purified LPLases described in Sections IV-1 and IV-2 there were differences in their some properties, the other properties were similar each other, so that it seemed that these enzymes were same. It was determined that this LPLase was classified into the reaction type of LPLase 1,2.

Chapter V;

LPLase has a very weak LPC-LPC acyltransferase activity that is less than one hundredth of LPLase activity. But, in one of purified LPLase preparations, it was observed that LPC-LPC acyltransferase activity increased with the number of days during the preservation in 50 mM phosphate buffer, pH 7.0 at 0°C after the storage at -20°C. At 30 days after thawing, the ratio of the reaction velocity of LPLase activity to LPC-LPC acyltransferase activity became 6 : 1. It was observed on disc electrophoresis that the mobility of LPLase which accompanied the increased LPC-LPC acyltransferase activity was different from that of LPLase in which LPC-LPC acyltransferase activity was before increase. The position of LPC-LPC acyltransferase activity on disc gel, however, coincided always with that of LPLase activity. Both activities could not be separated by isoelectric focusing and gel filtration column chromatography, and showed the same properties about an

optimal pH for the reaction, pH stability and the inhibition by metal ions. However, the substances, which not only bind to the enzyme protein but also change the dispersion state of the substrate, differently affected both activities.

From the results described in Chapter II through V, it is concluded that PLase A_1, PLase A_2, PLase B and LPLase 1,2 represent practically all of PLases produced by *C. centrifugum*. When total activity of each PLase in crude enzyme preparation was compared under its optimal condition, the total PLase A_1 activity was about 3.2-fold of the total PLase A_2 activity (Table II in Chapter II), and the total PLase B activity (Table I in Chapter III) was less than a few percent of the total PLase A activity (Table I in Chapter II). The total LPLase 1,2 activity (Table I in Chapter III) was much less than the total PLase A_1 activity (Table I in Chapter II), and the intramolecular isomerization of 2-acyl-lysophospholipids seemed to be little under the acidic condition which would be resulted from the oxalic acid produced by *C. centrifugum*. Although the enzyme assay conditions used in these experiments were artificial ones, it seems reasonable to conclude that PLase A_1 is the main enzyme of these PLases in natural system as well. The outline of the degradation of phospholipids by PLases produced by *C. centrifugum* is shown in Fig. 1.

When these PLases attack diacylphospholipids, it is expected that 2-acyl-lysophospholipids will be produced and accumulated.

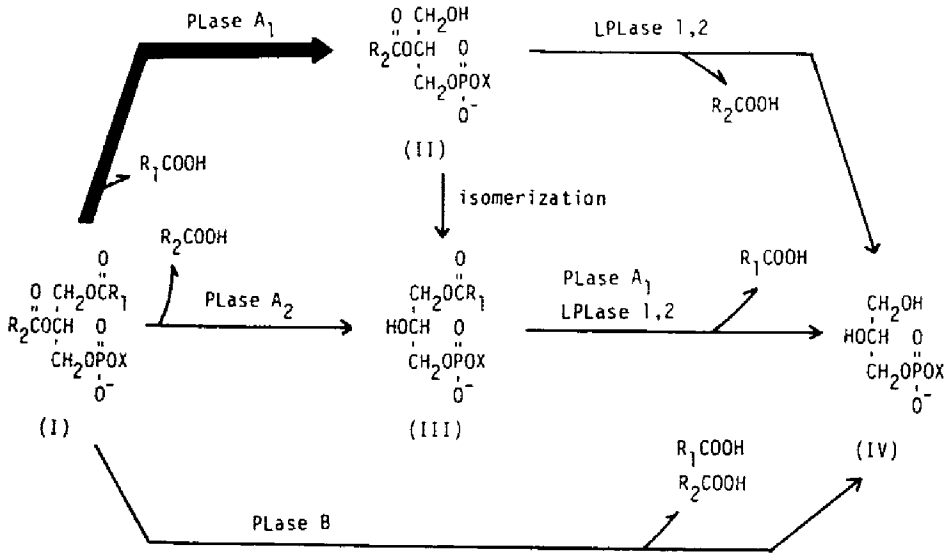


Fig. 1. Degradation of Phospholipids by Phospholipases Produced by *C. centrifugum*. R_1CO- and R_2CO-, fatty acyl residues; X, base moiety. (I), diacylphospholipids ; (II), 2-acyl-lysophospholipids ; (III), 1-acyl-lysophospholipids ; (IV), glycerylphosphoryl-bases.

In fact, when PC was hydrolyzed by the crude enzyme at pH 4.0 under the presence of 0.2% Triton X-100, much 2-acyl-LPC was accumulated.

Tseng *et al.*[7] supposed that in many plant-pathogen the enzymatic destruction of plant cell membranes would deduce a cell death, and assumed PLases and proteases which decompose the major constituents of cell membranes as candidative enzymes. It has been clarified in this study that PLase A_1 is the main enzyme in PLases produced by *C. centrifugum* as described above. It was expected that when PLases produced by *C. centrifugum* attacked the plant cell membranes in the course of its invasion upon plant tissues, various lysophospholipids, which were toxic because of the induction of lysis of membrane with a small amount, were abundantly accumulated in the membrane, and in consequence, the cell membranes were

disrupted and cell death was easily resulted. Until now, in *C. centrifugum*, the killing substance of cells has been simply assumed as the compound like organic acids[10,15-16] or degradative enzymes.[11] Thus, it seems to be an interesting hypothesis that the reaction product, which is toxic with a small amount, causes a cell death.

In the course of the study on the effect of different pretreatments on the extractability of lipid from yeast, Hasegawa *et al.*[9] found that the yeast phospholipids disappeared completely, accompanied by an increase of free fatty acids by lysis, with a cell wall lyser from *C. centrifugum*. They investigated the cause of this disappearance and found the presence of PLases A and B and LPLase in the cell wall lyser. In the present thesis, each PLase was purified from the culture broth of *C. centrifugum*, and its kind, enzymatic properties and possible degradative pathways of phospholipids were clarified.

As described in Chapter II, since PLase A_1 was produced abundantly and had a high positional specificity, this enzyme would be expected to become available corresponding to the snake venom PLase A_2 as an enzyme reagent or as a model enzyme for the elucidation of its reaction mechanism.

The significance of the existence of PLases in this mold was considered to lie in the lysis of the cell membrane of plant tissues and the decomposition of phospholipids as a source of nutrients for the mold.

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